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13. ABSTRACT (Maximum 200 words)

Our research supported by this contract has been directed along 3 main lines: 1) analysis of crosslinked hemoglobin samples (64,000 MW) from the U.S. Army Medical Research Detachment headed by Col. John Hess. This has been achieved by various protein separation methods under both native and denaturing conditions in order to monitor the product of the pilot plant. 2) studies on the preparation of higher molecular weight cross-linked hemoglobins (128,000 MW). Various bifunctional agents are being evaluated and 3) studies on recombinant hemoglobins to understand the molecular basis for the low oxygen affinity of certain hemoglobins as a basis for future blood substitute research.

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Introduction

In general, the scope of our research activities on this contract has been divided into three major related categories as described separately and completely in the body of this annual report:

1. Analysis of cross-linked hemoglobin samples from the new lab headed by Col. John Hess. This pilot plant was transferred from LAIR in San Francisco to Rockville, MD and set up there in the past six months. Operation of this pilot plant for cross-linked hemoglobins commenced in late 1994 and we started our analysis on hemoglobin samples either brought back by Manning or sent to Manning's lab. Manning also made several trips to Col. Hess's lab for discussion on the protocols. A continuing part of our contract with Dr. Hess is to continue to analyze samples in order to optimize the reaction conditions to obtain the highest yield possible.

2. These studies on the preparation of well-defined cross-linked hemoglobins greater than 64,000 MW were initiated and are an on-going part of our contract with Col. Hess. They have been underway since the beginning of the contract. Preliminary studies conducted here are aimed at producing pure and well-characterized 128,000 molecular weight and higher cross-linked hemoglobins for further plasma retention studies (initially on a laboratory scale and later on a large scale).

3. Continuation of studies of expression of recombinant human hemoglobins in yeast in order to understand the basis for low affinity hemoglobin mutants.

Experimental Methods:

Chromatography of Cross-linked Hb - For preparative purposes the crosslinked hemoglobin (total, 200-250 mg) was applied to a Whatman DE-52 column (2 x 30 cm) and eluted with a linear gradient of 50 mM Tris acetate from pH 8.3 to pH 6.3 (500 ml of each). For removal of the most adherent components, the column was further eluted with 500 ml of the pH 6.3 buffer.

Extent of Cross-linking - The amount of subunit crosslinking was determined by SDS/PAGE; the procedure of Laemmli was used with a 1 mm-thick, 12% crosslinked gel. The gel was stained with Coomassie blue for 1 hr and then destained in 30% (vol/vol) methanol/5% (vol/vol) acetic acid. The amount of crosslinking was estimated by densitometry on a Gilford model 2520 instrument equipped with a Shimadzu Chromatopac recorder model C-R6A.

Recombinant DNA Procedures

Site-directed mutagenesis and PCR - The plasmid pGS189 and pGS389 that contained the full-length human α- and β-globin cDNAs under transcriptional control of dual pGGAP promoter were used as gene sources. In our system the 1.2 kb-Xho1 fragment containing a single GGAP promoter amd the β-globin from pGS189 was inserted into the Xho1 site on the *E.coli* vector, pBluescript^R SK$^+$ with opposite transcriptional direction of *lac* promoter to create pBSK-BETA as a template for PCR mutagenesis. Two *Bam*H1 sites are presented at 10 nucleotides upstream of the mutated site (Asn at position 102th) and located at downstream of Xho1 site on the multiple cloning site encompassing the C-terminal region of β-globin gene, respectively.

Taking advantage of these *Bam*H1 sites, oligonucleotides containing a *Bam*H1 site and including mismatches were used as primer in the PCR methodology to generate amino acid substitutions in the amplified DNA fragment, using pBSK-BETA as template. The following mixed oligonucleotides 5'-CGTGGATCCTGAGXXXTTCAGGCTCCTG-3'; the site indicated as XXX was substituted by A/T. T/G.G/C for Met, Ile, Arg, Ser, Phe, Leu, Trp or Cys as the A mix primer, and by G/C.A/C.T/A for Asp, Glu, Ala, His or Pro as the B mix primer) were synthesized. The M13 reverse primer (5'-AACAGCTATGACCATG-3') was used as a primer for the other strand.

Approximately 1 μg of pBSK-BETA DNA was used for input, and amplification was carried out with 20 cycles of 1 min denaturation at 94°C, 2 min annealing at 37°C and 1.5 min extension at 72°C. The concentrations of A mix primer or B mix primer and M13 reverse primer, dNTP and Mg^{2+} in the reaction mixture were made according to the standard procedures suggested by Perkin-Elmer/Cetus.

The amplified DNA fragment (710 base pairs) was digested with *Bam*H1 and purified from agarose gel by using Pre A-gene kit (BioRad). The purified 580-bps *Bam*H1 fragments were subcloned back to the *Bam*H1 site of pBSK-BETA. The correct insertional direction of the mutated *Bam*H1 fragment in pBSK-BETA$^{(M)}$ was

determined by analysis with restriction enzymes Sal1 or Xho1, and then subjecting the digests to DNA sequence analysis. The mutation at the 102^{th} position of β-globin gene was confirmed by this procedure as well as the absence of additional changes introduced by the PCR procedure.

After complete DNA sequence confirmation, the plasmid pBSK-BETA$^{(M)}$ was treated with Xho1 to release the cassette with the mutated β-globin cDNA region, which was subsequently inserted into the expression vector pGS389 that had been digested with the same restriction enzyme. The correct orientation of the DNA fragment was again confirmed by restriction analysis and by DNA sequencing, and the recombinant plasmid pGS389-BETA$^{(M)}$ was transformed into Saccharomyces cerevisiae GSY112 cir^{o} strain. Transformants were selected on complete minimal agar plate without uracil.

Determination of Oxygen Binding Curves - For the recombinant and natural hemoglobins (10 μl, 2.4 mM in heme in 50 mM bis-Tris acetate, pH 7.5), the oxygen binding curves were measured at 37°C on a modified Hem-O-Scan instrument. Prior to the analysis, the CO form of Hb, the ligand state in which the mutant hemoglobin was purified to ensure that the heme was not oxidized, was converted to the oxy form by several exposures to incandescent light in an atmosphere of 100% O_2, as described previously. This conversion was considered complete when the A_{540}/A_{560} ratio was 1.7. The oxygen equilibrium curves of the recombinant and normal hemoglobins were measured in the absence or presence of 0.5 M chloride. The gas mixture used to achieve oxygenation was either 25% O_2/75% N_2, or 50% O_2/50% N_2.

CATEGORY 1

SUMMARY OF ANALYSES PERFORMED FOR U.S. ARMY LABS (COL. JOHN HESS)

Date	Sample	Analyses Performed
11/21/94	#94319 Bag 31	DE Column SDS-PAGE-scan Spectra
2/13/95	#94319	DE Col SDS-PAGE Spectra
2/8/95	#94319-"B" fraction	Superdex 75 FPLC
2/14/95	#94319-"B" fraction	Superose 12-FPLC
2/15/95	DBBF-B + DIBS + DMS + TMMP	Superose 12-FPLC
2/16/95	Conc DBBF-B + TMMP + DMS + Glycolaldehyde	SDS-PAGE Superose 12-FPLC
2/21/95	Conc DBBF-B+TMMP + DMS	SDS-PAGE Superose 12
2/23/95	DBBF-B + DMA DMS DMP DTBP	SDS-PAGE Superose 12
2/28/95	# 950207-00-X	DE52 Col SDS-PAGE Mono Q

Date	Lot	Analyses Performed
3/6/95	DBBF-B + DMS	SDS-PAGE Superose 12
3/7/95	DBBF-B + DMS at 3 temperatures	SDS-PAGE Superose 12
3/9/95	DBBF-B + DMS	SDS-PAGE
3/15/95	# 94319 Purification	DE Col SDS PAGE IEF
3/23/95	Lot # 950314-00-X also Lot 94319	SDS-PAGE Paragon Mono Q
4/26/95	Lot # 950404-00-X	mono-Q
5/25/95	Lot #950516-01-S Stromafree Lot #950516-02-Y	DE52 Col SDS-PAGE Mono-Q Paragon of DE Col. fractions

4/95: Melting Point Determinations on DBBF crosslinker itself

Abbreviations:

DE-Col = DEAE-cellulose chromatography
SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
FPLC = Pharmacia's liquid chromatography system
DBBF-Hb = dibromosalicyl fumarate derivative of Hb
"B" fraction = the desired α-α DBBF-crosslink product
TMMP = trimesoyl tris (methyl phosphate)
DMS = dimethyl suberimidate
DMA = dimethyl adipimidate
DMP = dimethyl pimelimidate

Purpose: These studies were performed on the various batches of cross-linked hemoglobins defined in the above table. By the various analyses listed we can calculate the extent of

crosslinking and the yield of the desired product having a single crosslink between the two Lys-99(α). In general, the procedures used are the same as those currently under study to obtain 128,000 and higher molecular weight defined crosslinked hemoglobin described next.

CATEGORY 2

2. <u>Preparation of Defined Cross-linked Hemoglobins of 128,000 molecular weight</u>

In this phase of the study we are starting with DBBF-Hb (64,000 molecular weight; crosslinked) from Col. John Hess. The initial attempts are to screen a number of crosslinking agents that would react with unessential amino groups on the outside of the 64,000 M.W. tetramers to cross-link two of these together to produce 128,000 MW tetramers.

We are currently investigating which cross-linker is best for that purpose and we are testing the following:

a) Diisothiocyanotobenzene sulfonate (DIBS), a crosslinker that we have studied previously in collaboration with Dr. Hess.

b) TMMP, a triple-crosslinker provided to us by Dr. Ronald Kluger of the University of Toronto.

c) Dimethyl suberimidate and related cross-linkers

d) Glycolaldehyde (Gly) a cross-linker that we have previously studied on previously uncross-linked Hb.

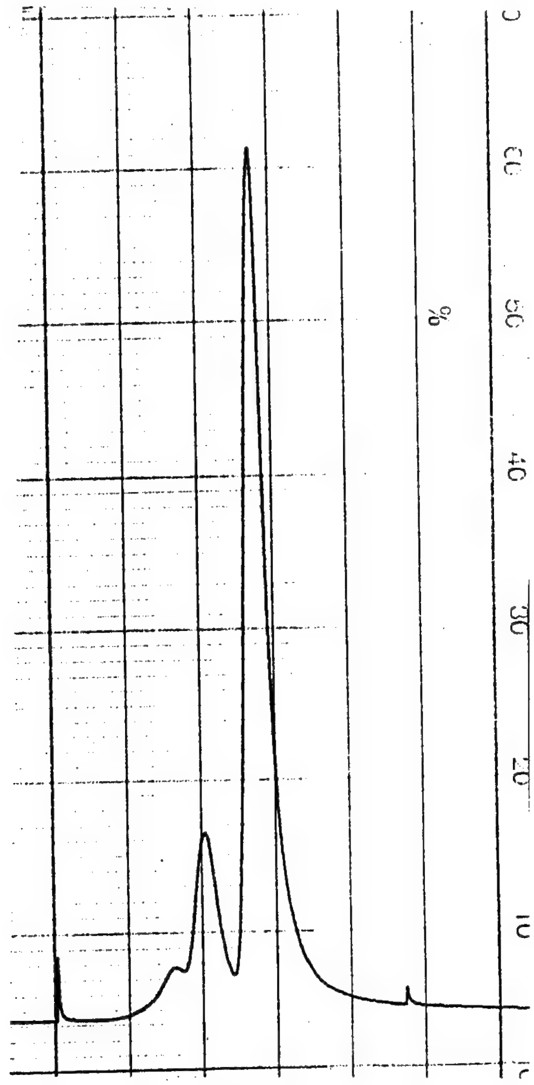
The reactions are being carried out for varying times and the samples are being analyzed by the criteria listed in the first table to find the best cross-linker and optimum conditions to obtain 128,000 M.W. hemoglobin.

The methods being employed are: SDS-PAGE followed by scanning of the gel by densitometry to determine the amount of cross-linking <u>between</u> <u>subunits</u> (i.e. intra-tetrameric) and FPLC with native samples to determine the amount of crosslinking <u>between</u> <u>tetramers</u> (i.e. inter-tetrameric).

Examples of the results to date are shown below as a representation of the current state of the research. We are continuing along this general line in order to obtain even higher yields.

SDS-PAGE Results of screening: (Crosslinking of Subunits, intra-tetrameric)

	% of Total			
	16K	32K	48K	64K
50 μM DBBF-B + 20 mM DMS 1 hr	20	30	22	28
200 μM DBBF-B + 20 mM DMS 1 hr	16	35	23	26
400 μM DBBF-B + 20 mM DMS 1 hr	25	40	21	14
600 μM DBBF-B + 20 mM DMS -1 hr	27	43	19	12



↑ 128K

FPLC Analysis (Native Hb)

	Native Molecular Weight			
	32K	64K	128K	>128K
350 μM DBBF-B + DMS 2 hr RT	0	91	9	0
350 μM DBBF-B + DMS 1 hr RT	0	96	4	0
200 μM DBBF-B + DMS 1 hr RT	0	88	12	0
400 μM DBBF-B + DMS 1 hr RT	0	79	17	5
600 μM DBBF-B + DMS 1 hr RT	0	79	17	5

CATEGORY 3

3. Recombinant Hemoglobin Expressed in Yeast

Two recombinant mutants have been expressed, purified, characterized and evaluated as described below. Reprints describing the work in full are enclosed.

a) Asp-99(β) → Lys (D99K(β))

Site-directed mutagenesis of an important subunit contact site, Asp-99(β), by a Lys residue (D99K(β)) was proven by sequencing the entire β-globin gene and the mutant tryptic peptide. Oxygen equilibrium curves of the mutant hemoglobin (Hb) (2-15 mM in heme) indicated that it had an increased oxygen affinity and a lowered but significant amount of cooperativity compared to native HbA. However, in contrast to normal HbA, oxygen binding of the recombinant mutant Hb was only marginally affected by the allosteric regulators 2,3-diphosphoglycerate or inositol hexaphosphate and was not at all responsive to chloride. At concentrations of 0.2 mM or lower in heme, the mutant D99K(β) Hb was predominantly a dimer as demonstrated by gel filtration, haptoglobin binding, fluorescence quenching, and light scattering. The purified dimeric recombinant Hb mutant exists in 2 forms that are separable on isoelectric focusing by about 0.1 pH unit, in contrast to tetrameric hemoglobin, which shows 1 band. These mutant forms, which were present in a ratio of 60:40, had the same masses for their heme and globin moieties as determined by mass spectrometry. The elution positions of the α- and β-globin subunits on HPLC were identical. Circular dichroism studies showed that one form of the mutant Hb had a negative ellipticity at 410 nm and the other had positive ellipticity at this wavelength. The findings suggest that the 2 D99K(β) recombinant mutant forms have differences in their heme-protein environments.

b) Asn-102(β) → Ala (N102A(β))

A recombinant mutant hemoglobin with Asn-102(β) replaced by an Ala (N102A(β)) has been prepared by PCR amplification of a mutagenic DNA fragment and expression of the recombinant protein in yeast. The side chain of Asn-102(β) is part of an important region of the $\alpha_1\beta_2$ interface that undergoes large structural changes in the transition between the deoxy and oxy conformations. Three natural mutant Hbs with neutral substitutions of Thr, Ser, or Tyr at this site have low oxygen affinities because a hydrogen bond between Asn-102(β) and Asp-94(α) in normal HbA was considered to be absent in these mutants, thereby destabilizing the oxy conformation in favor of the deoxy conformation. This proposal has been tested by expression of an Hb containing alanine at position 102(β); alanine was chosen because its methyl side chain cannot participate in hydrogen bond formation, yet it is small enough not to disrupt the subunit

interface. The nature of the desired replacement was established by sequencing the entire mutated β-globin gene as well as the tryptic peptide containing thw substitution. Further characterization by SDS-PAGE, isoelectric focusing, HPLC analysis, mass spectrometry, amino acid analysis, and sequencing of the mutant tryptic peptide confirmed the purity of the rHb. Its oxygen binding curve (2.4 mM in heme) in the absence of chloride showed that it had a very low oxygen affinity with a P_{50} of 42 mm Hg. In the presence of added chloride (0.5 M), its oxygen affinity was further reduced only slightly to a P_{50} of 49 mm Hg. In contrast, the oxygen affinity of HbA was lowered two- to threefold by the same concentration of chloride. Comparison of the properties of N102A(β) with those of the D99K(β) Hb, substituted by a Lys (described above), demonstrates how the judicious choice of the amino acid substitution based upon the properties of natural mutations at a particular site can further enhance our understanding of the role of certain amino acid side chains in Hb function.

Conclusions

1. After setting up the pilot plant in Col. Hess's laboratory, it was then necessary to re-establish the optimum conditions for obtaining the desired cross-linked hemoglobin (referred to as Fraction "B" above). We employed the various analytical methods described in the first table to help Col. Hess and his colleagues do so. The plant is now fully functional to produce a cross-linked 64,000 MW hemoglobin. We continually check the various batches produced there to ensure their quality and yield.

2. Our next goal is to achieve experimental conditions whereby a 128,000 MW (and eventually 256,000 MW) cross-linked hemoglobin can be obtained. At the proper time the conditions at the pilot plant will be adjusted in order to produce such material on a large scale for testing.

3. The study of recombinant hemoglobins permits us to investigate the molecular mechanisms for the low oxygen affinity of hemoglobins. The objective is to produce such hemoglobins on a large scale and these may eventually become the products of the future.

Publications

1. Yanase, H., Cahill, S., Martin de Llano, J.J., Manning, L.R., Schneider, K., Chait, B.T., Vandegriff, K.M., Winslow, R.M., and Manning, J.M. (1994) Properties of a recombinant human hemoglobin with aspartic acid 99(β), an important intersubunit contact site, substituted by lysine. *Protein Sci.* **3**: 1213-1223.

2. Yanase, H., Manning, L.R., Vandegriff, K., Winslow, R.M., and Manning, J.M. (1995) A recombinant human hemoglobin with asparagine-102(β) substituted by alanine has a limiting low oxygen affinity, reduced marginally chloride. *Protein Sci.* **4**: 21-28.

3. Manning, J.M. (1995) Design of Chemically Modified and Recombinant Hemoglobins as Potential Red Cell Substitutes. In: Blood Substitutes: Physiological Basis of Efficacy (Winslow, R.M., Vandegriff, K.D., and Intaglietta, M., Eds.), Birkhauser. Chapter 6, pgs. 76-89.

(Copies of each are enclosed)

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Ms. Wanda Jones (Research Technician)

Graduate Degrees:

None

Protein Science (1995), *4*:21–28. Cambridge University Press. Printed in the USA.

A recombinant human hemoglobin with asparagine-102(β) substituted by alanine has a limiting low oxygen affinity, reduced marginally by chloride

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(RECEIVED August 8, 1994; ACCEPTED October 12, 1994)

Abstract

A recombinant (r) mutant hemoglobin (Hb) with Asn-102(β) replaced by an Ala (N102A(β)) has been prepared by PCR amplification of a mutagenic DNA fragment and expression of the recombinant protein in yeast. The side chain of Asn-102(β) is part of an important region of the $\alpha_1\beta_2$ interface that undergoes large structural changes in the transition between the deoxy and oxy conformations. Three natural mutant Hbs with neutral substitutions of Thr, Ser, or Tyr at this site have low oxygen affinities because a hydrogen bond between Asn-102(β) and Asp-94(α) in normal HbA was considered to be absent in these mutants, thereby destabilizing the oxy conformation in favor of the deoxy conformation. This proposal has been tested by expression of an rHb containing alanine at position 102(β); alanine was chosen because its methyl side chain cannot participate in hydrogen bond formation, yet it is small enough not to disrupt the subunit interface. The nature of the desired replacement was established by sequencing the entire mutated β-globin gene as well as the tryptic peptide containing the substitution. Further characterization by SDS-PAGE, isoelectric focusing, HPLC analysis, mass spectrometry, amino acid analysis, and sequencing of the mutant tryptic peptide confirmed the purity of the rHb. Its oxygen binding curve (2.4 mM in heme) in the absence of chloride showed that it had a very low oxygen affinity with a P_{50} of 42 mm Hg. In the presence of added chloride (0.5 M), its oxygen affinity was further reduced only slightly to a P_{50} of 49 mm Hg. In contrast, the oxygen affinity of HbA was lowered two- to threefold by the same concentration of chloride. Comparison of the properties of the rHb N102A(β) with those of the rHb with Asp-99(β), which is at this same subunit interface, substituted by a Lys (Yanase H, et al., 1994, *Protein Sci 3*:1213–1223), demonstrates how the judicious choice of the amino acid substitution based upon the properties of natural mutations at a particular site can further enhance our understanding of the role of certain amino acid side chains in Hb function.

Keywords: chloride effect; hemoglobin; low oxygen affinity; mutagenesis

Hemoglobin (Hb) Kansas is a naturally occurring mutant Hb in which asparagine at position 102 of the β-chain is replaced by a threonine residue (Bonaventura & Riggs, 1968; Ogawa et al., 1972; Gibson, 1973; Atha & Riggs, 1976; Atha et al., 1979). This mutation occurs in an important region of the $\alpha_1\beta_2$ interface, which undergoes large rearrangements in the transition between the oxy and deoxy conformations of Hb (Perutz & TenEyck, 1971; Perutz et al., 1976; Perutz, 1990) (Fig. 1). It has been proposed that Hb Kansas has a low oxygen affinity because the hydrogen bond that usually occurs between Asn-102(β) and Asp-94(α) in normal Hb to stabilize the oxy conformation is absent in this mutant Hb, thus shifting the equilibrium toward the deoxy conformation (Greer, 1971). Two other natural mutants with neutral substitutions at this position and also with low oxygen affinities are Hb Beth Israel (Asn-102(β) $\rightarrow$ Ser) (Nagel et al., 1976) and Hb St. Mande (Asn-102(β) $\rightarrow$ Tyr) (Arous et al., 1981; Poyart et al., 1990). However, in these three natural mutant Hbs, each with hydroxyl-bearing substitutions, hydrogen bonding remains a possibility and indeed has been found for Hb St. Mande (Poyart et al., 1990). Therefore, the question arose

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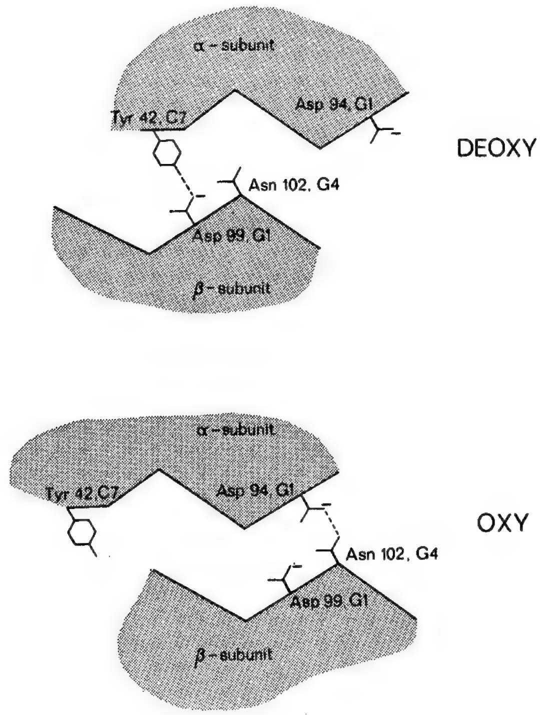

Fig. 1. Segment of the $\alpha_1\beta_2$ interface. From Perutz and Ten Eyck (1971).

whether there were alternative mechanisms for the low oxygen affinity of these mutants. Hence, in this communication, we report the effects of the substitution of an Ala residue at position 102(β) on the functional properties of Hb. We also compare its drastically different properties with those of a recombinant (r) Hb in which Asp-99(β) at an adjacent site in the same $\alpha_1\beta_2$ interface was substituted by a Lys (Yanase et al., 1994) (Fig. 1).

A fourth natural mutant at position 102, Hb Richmond, has a Lys instead of an Asn residue at this site (Efremov et al., 1969). Winslow and Charache (1975) found that the oxygen affinity of Hb Richmond was higher at low pH and lower at high pH compared to HbA, with a crossover point near physiological pH. According to Greer (1971), Lys-102(β) in Hb Richmond can form a salt bridge with Asp-94(α) in the oxy conformation.

To express rHb, we employ a yeast plasmid that contains both human α- and β-globin genes on the same plasmid (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b). This expression system uses endogenous yeast heme to produce a soluble Hb tetramer that is processed at its N-terminus and has the same conformation and functional properties as natural human Hb isolated from human red cells, as demonstrated by a variety of techniques (Martin de Llano et al., 1993a, 1993b). In addition, it has recently been shown that two rHb expressed in this system have the same CD spectra as natural Hb from 200 to 650 nm, a range that covers both overall protein conformation and the interaction with heme (Martin de Llano & Manning, 1994). Thus, this yeast expression system is ideal to study the effects of specific changes at important regions of either the α- or β-chains in order to gain a fuller appreciation of the details of Hb function.

Results

Identification of the mutation on the β-globin gene

Using PCR-assisted mutagenesis, we prepared mutants to express various substitutions at position 102 of the β-chain of Hb. The recombinant plasmids pBSK-BETA$^{(M)}$, which were constructed to insert the amplified *Bam* H1 fragment with the mutagenic A or B mix primer, were identified by DNA sequencing as having a mutated base at position 102. Eleven β-globin mutants were isolated – N102A, N102L, N102R, N102S, N102F, N102D, N102E, N102H, N102P, N102M, and the double mutant N102W/F103I. Of these mutant β-globin genes, pBSK-BETA^{N102A} was selected because of the small size and neutral nature of its side chain, and the excised mutant β-globin gene was subcloned into pGS389 to construct pGS389 N102A(β). Plasmid pGS389 N102A(β) was transformed into the *Saccharomyces cerevisiae* GSY112 strain and the rHb N102(β) was expressed in the yeast system by the procedures described previously (Martin de Llano et al., 1993a, 1993b).

Purification of the N102A(β) rHb mutant

The growth of the yeast was not affected by the expression of this rHb mutant as previously found for other Hbs expressed in this system (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). The rHb, which was purified first by batchwise chromatography on CM-52 and then by HPLC as described above, eluted as a single, symmetrical peak on the Synchropak CM 300 column (Martin de Llano et al., 1993a).

Isoelectric focusing

Analysis of the purified recombinant Hb by isoelectric focusing between pH 6 and 8 showed that it migrated as a single tight band in a position slightly cathodal to that of HbA (Fig. 2). This difference in pI was unexpected considering the neutral nature of the substitution of an Ala for an Asn, but anomalous chromatographic behavior of Hb Kansas and of Hb St. Mande have been noted previously by Bonaventura and Riggs (1968) and by Poyart et al. (1990), respectively.

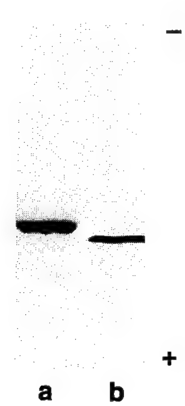

Fig. 2. Isoelectric focusing of N102A(β) mutant Hb. A gel (pH 6–8) (Isolab) was electrophoresed at 10 W for 40 min and stained with 0.2% bromphenol blue. Lane a: N102A(β) mutant Hb; lane b: a natural Hb.

Separation of α- and β-chains

The purified recombinant N102A(β) Hb was subjected to HPLC analysis under denaturing conditions in order to separate and analyze the α- and β-globin chains. The mutant β-chain eluted at 28.5 min, significantly later than the β-chain from natural HbA (25.0 min) (Fig. 3). This result is consistent with the more hydrophobic nature of the mutant peptide where Ala is substituted for Asn. The α-chains from the recombinant mutant and the normal Hb eluted in the same positions within experimental error (31.2 min and 32.0 min, respectively), consistent with the absence of a substitution in the α-chain. Based on the significant differences in the values for serine, glutamic acid, glycine, alanine, and valine between the α- and β-chains, the results of the amino acid analysis permitted their assignments (data not shown).

Peptide mapping

The mutant β-chain obtained as described in Figure 3 was reduced and carboxymethylated as described by Crestfield et al. (1963) and then digested with trypsin, as described previously (Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). The digest was applied to a Vydac C-18 column and eluted with the gradient described below. Comparison of the pattern of the tryptic peptide map of the mutant β-globin chain with the corresponding tryptic map of the normal β-globin chain showed one major peak present in the mutant β-chain (Fig. 4, open arrow) that was absent in the tryptic peptide map of normal β-globin, which itself had a peptide (Fig. 4, closed arrow) that was absent in the mutant Hb. The late elution of the mutant peptide compared with the corresponding peptide from normal Hb was consistent with its increased hydrophobicity. The remainder of the peptide map (not shown) was identical for the two β-globin chains.

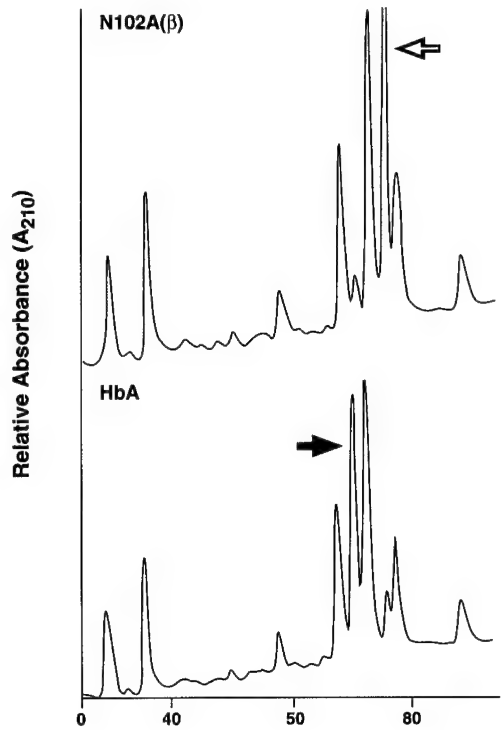

Fig. 4. Tryptic peptide maps of β-chain of N102A(β) mutant and a natural Hb. The β-chains of each Hb were isolated on a Vydac C-4 column as shown in Figure 3, then reduced, carboxymethylated, and digested with *N*-tosyl-L-phenylalanine chloromethyl-trypsin, and chromatographed on a Vydac C-18 column as described in the text. The closed and open arrows indicate unique peaks in digests of natural and mutant β-globin chains, respectively. The peptide in the upper peak (open arrow) from mutant Hb was hydrolyzed and then subjected to protein sequencing.

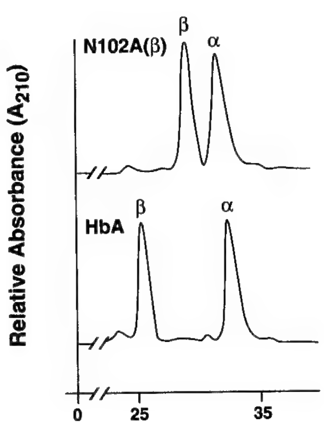

Fig. 3. Separation of α- and β-globin chains of N102A(β) mutant and of natural HbA. The mutant and natural Hb were purified by HPLC and then chromatographed on a Vydac C-4 column as described in the text. **A:** Elution profile of N102A(β) mutant (100 μg). **B:** Elution profile of natural Hb (115 μg).

Sequence of the mutant peptide

The unique tryptic peptide isolated from the mutant β-chain, which was purified with a yield of 32.3% as described above, was subjected to amino acid sequencing. The results showed that this peptide comprised amino acid residues 83–104 of the β-chain and Ala was found in high yield at position 102 (Fig. 5), confirming the nature of the substitution. In this peptide, trypsin did not cleave the peptide bond after Lys-95(β) because of the presence of Asp at position 94.

Mass spectrometric analysis

The purified native recombinant N102A(β) tetramer was subjected to mass spectrometric analysis as described in the Materials and methods. The results (Table 1) indicated that the mass of the recombinant β-chain was consistent with the calculated value of 15,825, which is 43 mass units lower than a normal β-chain, exactly that calculated for the replacement of an Asn by an Ala. The mass of the α-chain was 15,126, which is the same as the mass of the normal α-chain from human HbA.

Gly[83]	Thr[84]	Phe[85]	Ala[86]	Thr[87]	Leu[88]	Ser[89]	Glu[90]	Leu[91]	His[92]	Cys[93]	Asp[94]	Lys[95]	Leu[96]
446	190	363	357	142	293	81	168	211	114	-	131	123	146

His[97]	Val[98]	Asp[99]	Pro[100]	Glu[101]	<u>Ala</u>[102]	Phe[103]	Arg[104]
74	121	65	83	46	65	52	24

Fig. 5. Amino acid sequence of mutant tryptic peptide from N102A(β) mutant Hb. The tryptic peptide that was sequenced was isolated as described in Figure 4. The numbers under the sequence are the repetitive yields at each cycle of Edman degradation. Cys-93 was present as the *S*-carboxymethyl derivative after treatment with iodoacetate as described in the text. The underlined Ala at position 102 is the site of the mutation.

Spectral properties of recombinant N102A(β) mutant Hb

The spectrum of the mutant Hb in the ultraviolet and visible range indicated that the ratios of the peak absorbances were practically identical to those of normal HbA (data not shown).

Tetramer–dimer dissociation

The extent of dimer formation of the mutant Hb was compared to that of normal HbA by two procedures, i.e., filtration of the haptoglobin–Hb dimer complex (Table 2) and quenching of haptoglobin fluorescence upon binding of Hb dimers (Fig. 6). The results found by these two techniques were in agreement and indicated that in the micromolar concentration range, the N102A(β) mutant in both the oxy and deoxy conformations behaved in a manner practically identical to that of HbA. The small amounts of dimer found for both the normal and recombinant mutant Hb under deoxygenated conditions were likely due to the slow displacement of the tetramer–dimer equilibrium toward dimer formation induced by the presence of haptoglobin.

Oxygen binding properties

A P_{50} value of 42 mm Hg was found for the recombinant mutant N102A(β) Hb (Fig. 7; Table 3). Because oxygenation rate was slow for this low-affinity mutant, we changed the usual gas mixture containing 25% O_2 to one containing 50% O_2. It is especially important to vary this parameter with low-affinity mutants. Another consideration in obtaining accurate values is the actual extent of oxygen saturation attained. For example, for a particular turtle Hb with a low oxygen affinity, Lapennas et al. (1981) calculated that the observed P_{50} value differed from the true value by as much as 5 mm Hg, depending on the value taken for 100% saturation. In our studies comparing the effects of 25% O_2 with 50% O_2, we found that, although P_{50} values were lower with the latter gas mixture, the degree of response to added chloride did not change significantly (Table 3), i.e., the oxygen affinity of HbA was lowered more than twofold by addition of chloride, but the oxygen affinity of the N102A(β) mutant Hb was decreased by only 10–20% and these values did not vary with either 25% O_2 or 50% O_2. The results suggest that the major contribution to the lowering of the oxygen affinity was due to the substitution itself and not to added chloride. It is important to point out that other recombinant mutant Hb expressed in this same yeast system, i.e., HbS, interacted normally with chloride in terms of a lowered oxygen affinity (Martin de Llano et al., 1993a, 1993b).

Table 1. *Mass spectrometric analysis of the N102A(β) mutant Hb*

	Mass
α-Globin	
Measured	15,126.8
Calculated	15,126.4
β-Globin	
Measured	15,826.1
Calculated	15,825.2

Discussion

The extremely low oxygen affinity of the recombinant N102A(β) mutant Hb is consistent with the general trend reported for the natural mutants with hydroxyl-containing neutral substitutions at this position, i.e., Hb Kansas (Asn → Thr), Hb Beth Israel (Asn → Ser), and Hb St. Mande (Asn → Tyr). However, the magnitude of the lowering of the oxygen affinity of the recombinant mutant Hb N102A(β) was significantly greater than that of any of these three natural mutants. The results suggest a nearly complete shift in the deoxy–oxy equilibrium toward the deoxy conformation due to the lack of any interaction between the side

Table 2. *Extent of dimer formation of HbA and the N102A(β) recombinant mutant as determined by haptoglobin binding*[a]

	% Dimer
Oxy HbA	74
Oxy N102A(β)	67
Deoxy HbA	14
Deoxy N102A(β)	14

[a] The Hb concentrations were 0.5 μM as tetramer and the haptoglobin concentrations were 1.0 μM. The mixing and the filtration with the Centricon apparatus were performed as described previously (Yanase et al., 1994). When present, the sodium dithionite concentration was 0.6 M.

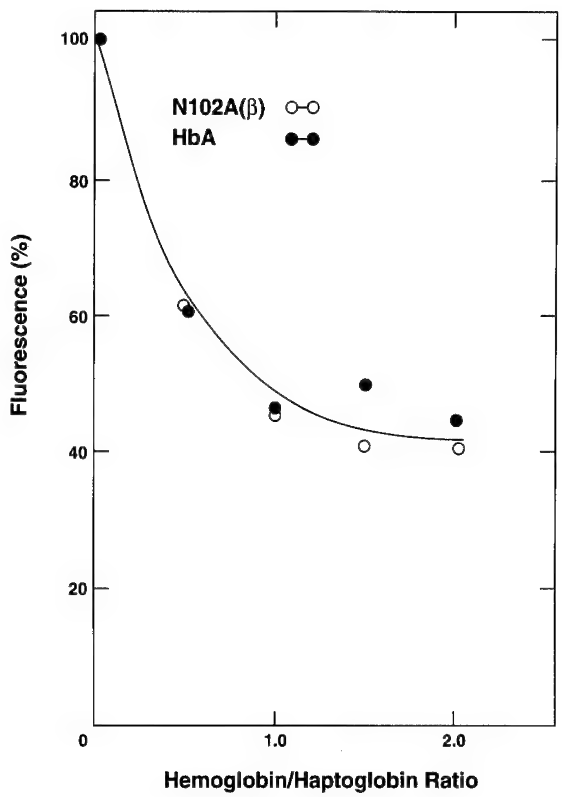

Fig. 6. Quenching of haptoglobin fluorescence by Hb dimers. The quenching of haptoglobin fluorescence by binding of Hb dimers under oxygenated conditions was determined as described in the text for various ratios of Hb to haptoglobin shown in the figure.

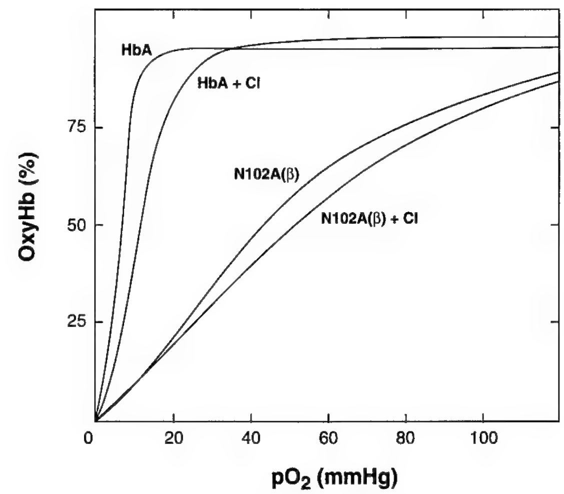

Fig. 7. Oxygen-binding curves of N102A(β) mutant and natural HbA. The oxygen equilibrium curves were determined at 37 °C on Hb (2.4 mM in heme) in 50 mM bis-Tris acetate, pH 7.5. The oxygenating gas contained 50% O_2/50% N_2. HbA attained complete oxygenation in less than 5 min, but the N102A(β) mutant required 15–20 min (that part of the curve not shown). The effect of chloride ion on the oxygen-binding affinity of Hb was determined in the presence of 0.5 M NaCl (Cl) as described in the text.

chains of Ala-102(β) and Asp-94(α). This argument implies that some hydrogen bond interaction still exists between the β-hydroxyl groups of the neutral natural mutants and Asp-94(α) in the oxy conformation. Indeed, in Hb St. Mande, Poyart et al. (1990) found evidence for a hydrogen bond between the phenolic group of Tyr-102(β) and Asp-94(α) in the oxy conformation, and also involving Tyr-42(α) in the deoxy conformation.

The very modest further reduction in oxygen affinity upon addition of chloride to the recombinant N102A(β) mutant Hb suggests that the limiting oxygen affinity may have already been nearly reached even in the absence of allosteric regulators. In contrast, Hb St. Mande still responds to chloride, but with a reduced efficiency compared to HbA (Poyart et al., 1990).

The natural mutant Hb with substitutions at position 102(β) have an increased tendency to dissociate to dimers in their oxygenated states (Bonaventura & Riggs, 1968; Ogawa et al., 1972; Gibson, 1973; Atha & Riggs, 1976; Atha et al., 1979). Thus, the dissociation constant of Hb Kansas in its oxygenated state is in the millimolar range, about 50 times greater than that for oxy HbA, but in its deoxygenated state, Hb Kansas dissociates to the same extent as normal HbA. This property plays a role in the determination of its functional properties. For example, an important difference in the studies with Hb Kansas and with the N102A(β) recombinant mutant is the Hb concentrations used for measurement of their oxygen binding curves. In the present study, relatively concentrated solutions (millimolar range in heme) of N102A(β) were used for the oxygen binding studies; at these concentrations, there is little dissociation into dimers in either the oxy or the deoxy conformations because the extent of dimerization was found to be very similar to that of HbA (in the micromolar concentration range). Even if there was some dissociation of the oxygenated recombinant Hb during measurement of the oxygen binding curve, it is unlikely that it occurred to a significant extent because a low oxygen affinity and signif-

Table 3. *Oxygen binding parameters for HbA and N102A(β) Hb*[a]

	No NaCl	0.5 M NaCl	25% O_2	50% O_2
HbA				
P_{50}[b] (mm Hg)	5	11		
n[b]	2.4	2.4		
P_{50} ratio[c] ($+Cl^-/-Cl^-$)			2.4	2.2
N102A(β)				
P_{50}[b] (mm Hg)	42	49		
n[b]	1.9	1.7		
P_{50} ratio[c] ($+Cl^-/-Cl^-$)			1.1	1.2

[a] P_{50} values are expressed in mm Hg at the point of 50% saturation. For the N102A(β) mutant, the P_{50} value was calculated from the oxygen equilibrium curve as shown in Figure 7 after approximately 90% O_2 saturation was attained in about 15–20 min. Cooperativity (n value) was calculated from the log–log plot of the saturation plot. The determinations were made on a modified Hem-O-Scan instrument at a Hb concentration of 2.4×10^{-3} M in heme and 37 °C.

[b] Determined using 50% O_2/50% N_2 as the oxygenating gas.

[c] Measurements were made separately with oxygenating gases containing either 25% O_2/75% N_2 or 50% O_2/50% N_2.

icant cooperativity were attained and these properties are not consistent with extensive dimerization (Turner et al., 1992). In contrast, the reported oxygen binding measurements for Hb Kansas were performed at dilute, i.e., micromolar Hb concentrations where there is extensive dissociation in the oxygenated state and loss in cooperativity. However, the natural mutant Hb St. Mande exhibits some cooperativity (Poyart et al., 1990). The results of the present study, as well as the previous report on the D99K(β) rHb (Yanase et al., 1994) indicate an advantage in using relatively high Hb concentrations for determination of the oxygen dissociation curves. Winslow et al. (1994) have also stressed the advantages of working with concentrated solutions of Hb to determine oxygen binding curves.

The oxygen binding properties of the N102A(β) mutant are in marked contrast to those found for the Asp-99(β) $\rightarrow$ Lys mutant (D99K(β)) (Yanase et al., 1994), which is at an adjacent site in the $\alpha_1\beta_2$ interface (see Jones et al. [1967] for a description of the properties of Hb Yakima, a natural human muatnt at position 99(β)). Thus, in HbA, Asp-99(β) site is linked to Tyr-42(α) by a hydrogen bond in the deoxy conformation but not in the oxy conformation (Fig. 1). The results with the D99K(β) rHb were interpreted to indicate that substitution by a Lys disfavored the deoxy conformation by interfering with the normal linkage at this site in HbA. Thus, the large and unfavorable Lys replacement led to significant disruption of the $\alpha_1\beta_2$ interface and extensive dimerization in both the oxy and deoxy conformations, at least in the micromolar heme concentration range in which the studies were performed. In contrast, the choice of Ala for the substitution in the N102A(β) rHb was designed to preserve the integrity of this subunit interface; this objective was realized by the finding that the N102A mutant in the deoxy conformation was tetrameric under the conditions used for similar measurements for deoxy HbA.

Neither rHb D99K(β) nor rHb N102A(β) had a significant response to chloride with respect to a lowered oxygen affinity, but for completely different reasons. In the case of the D99K(β) mutant, the tendency to form dimers precluded its extensive functional interaction with chloride because the tetrameric structure, which is a prerequisite for such binding (Perutz et al., 1976, 1994; Bonaventura & Bonaventura, 1978; Manning et al., 1978; Perutz, 1990; Ueno & Manning, 1992; Ueno et al., 1993; Bonaventura et al., 1994; Manning, 1994), is disfavored in this recombinant mutant. Thus, there are three major functional chloride binding regions—the N-terminal/C-terminal region of the α-chains, the DPG pocket, and the central dyad axis. The latter region connects the first two regions at either end of the tetramer. The N102A(β) mutant is completely tetrameric in the millimolar concentration range used for the oxygen equilibrium studies, and the results suggest that the R $\rightarrow$ T equilibrium is shifted almost entirely to the T state even in the absence of chloride; hence, addition of chloride has little further effect.

Shih et al. (1985) reported a comprehensive study of the oxygen binding properties and effects of allosteric regulators of the natural mutant Hb with various substitutions at position 101 of the β-chain, between positions 99(β) and 102(β). They concluded that the size and the nature of the charge of the substitution were most important in determining the oxygen affinities of the mutant Hb. Our findings on the functional properties of the recombinant mutants D99K(β) and N102A(β), for which the amino acid replacements were chosen with specific objectives in mind, are in agreement with their conclusions.

Materials and methods

Reagents

All reagents were analytical grade. Restriction endonucleases and modifying enzymes (T4 ligase, etc.) were purchased from Boehringer Mannheim and Gene AmpR PCR reagent kits were from Perkin-Elmer/Cetus (Norwalk, Connecticut). The nucleotides used to make the mutation were purchased from Operon Technologies (Alameda, California).

Site-directed mutagenesis and PCR

The plasmid pGS189 and pGS389 that contained the full-length human α- and β-globin cDNAs under transcriptional control of the dual pGGAP promoter were used as gene sources (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). Adachi et al. (1993) employed a different strategy to obtain the sickle Hb mutation in this system. In our system, the 1.2-kb *Xho* 1 fragment containing a single GGAP promoter and the β-globin from pGS189 was inserted into the *Xho* 1 site on the *Escherichia coli* vector, pBluescriptR SK+, with opposite transcriptional direction of the *lac* promoter to create pBSK-BETA as a template for PCR mutagenesis. Two *Bam*H 1 sites are presented at 10 nucleotides upstream of the mutated site (Asn at position 102th) and located downstream of the *Xho* 1 site on the multiple cloning site encompassing the C-terminal region of the β-globin gene, respectively.

Taking advantage of these *Bam*H 1 sites, oligonucleotides containing a *Bam*H 1 site and including mismatches were used as primers in the PCR methodology to generate amino acid substitutions in the amplified DNA fragment, using pBSK-BETA as template. The following mixed oligonucleotides were prepared: 5′-CGTGGATCCTGAGXXXTTCAGGCTCCTG-3′; the site indicated as XXX was substituted by A/T.T/G.G/C for Met, Ile, Arg, Ser, Phe, Leu, Trp, or Cys as the A mix primer, and by G/C.A/C.T/A for Asp, Glu, Ala, His, or Pro as the B mix primer were synthesized. The M13 reverse primer (5′-AACAG CTATGACCATG-3′) was used as a primer for the other strand.

Approximately 1 μg of pBSK-BETA DNA was used for input, and amplification was carried out with 20 cycles of 1 min denaturation at 94 °C, 2 min annealing at 37 °C, and 1.5 min extension at 72 °C. The concentrations of A mix primer or B mix primer and M13 reverse primer, dNTP, and Mg^{2+} in the reaction mixture were made according to the standard procedures suggested by Perkin-Elmer/Cetus.

The amplified DNA fragment (710 bp) was digested with *Bam*H 1 and purified from agarose gel by using a Pre A-gene kit (BioRad). The purified 580-bp *Bam*H 1 fragments were subcloned back to the *Bam*H 1 site of pBSK-BETA. The correct insertional direction of the mutated *Bam*H 1 fragment in pBSK-BETA(M) was determined by analysis with restriction enzymes *Sal* 1 or *Xho* 1, and then by subjecting the digests to DNA sequence analysis. The mutation at the 102nd position of the β-globin gene was confirmed by this procedure as well as the absence of additional changes introduced by the PCR procedure.

After complete DNA sequence confirmation, the plasmid pBSK-BETA(M) was treated with *Xho* 1 to release the cassette with the mutated β-globin cDNA region, which was subsequently inserted into the expression vector pGS389 that had been digested with the same restriction enzyme. The correct orienta-

tion of the DNA fragment was again confirmed by restriction analysis and by DNA sequencing, and the recombinant plasmid pGS389-BETA(M) was transformed into *S. cerevisiae* GSY112 ciro strain. Transformants were selected on complete minimal agar plates without uracil.

Bacterial and yeast strains and growth conditions

The yeast expression system that contains the human α- and β-globin genes on the same plasmid was used. The yeast strains were grown in 10 culture flasks (2 L each) in the presence of ethanol as the carbon source. Expression of Hb was induced by the addition of 2% galactose for 24 h (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994).

Protein purification

Upon completion of growth, the yeast cells were saturated with CO gas, collected, broken by homogenization in a Bead-Beater in the presence of glass beads, and the initial purification step was achieved on carboxymethyl-cellulose (Whatman, CM-52). The second and final purification step was on HPLC with a Synchropak CM 300 (250 × 10 mm) column (Martin de Llano et al., 1993a, 1993b). The gradient was modified so that it consisted of 20–70% buffer B for 10 min, 70–88% buffer B for 30 min, and then 13 min of the 100% buffer B, each at a flow rate of 2.5 mL/min. The compositions of the buffers were A = 30 mM bis-Tris, 30 mM sodium acetate, 1 mM EDTA, pH 6.4; B = 30 mM bis-Tris, 150 mM sodium acetate, 1 mM EDTA, pH 6.6. All buffers were saturated with CO.

Analytical procedures

SDS-PAGE was performed on the recombinant N102A(β) mutant, as described previously (Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). Electrophoresis of the native protein was done on the Beckman Paragon system. Globin chains were separated by HPLC on a Vydac C-4 column (250 × 4.6 mm), also described previously, except that a different gradient was used, i.e., 47–50% B for 6 min, 50–60% B for 60 min, and then 60–70% B for 10 min, each at a flow rate of 1.0 mL/min (A = 0.1% trifluoroacetic acid [TFA]; B = 80% acetonitrile, 0.1% TFA). Isoelectric focusing was performed on the pH 6–8 Hb-Resolve system from Isolab.

Amino acid analysis of globin chains isolated by this procedure was performed on a Beckman 6300 instrument with System Gold data handling system. Tryptic peptide mapping was performed on the reduced, carboxymethylated globin chains, and separation of the tryptic peptides was carried out by HPLC, as described previously (Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). However, the gradient used for separation of the tryptic peptide of the mutant β-chain was changed to 0–15% B for 10 min, 15–55% B for 120 min, and then 55–100% B for 10 min, each at a flow rate of 1.0 mL/min on a Vydac D-18 column. Sequencing of this peptide was performed on an Applied Biosystem gas phase sequencer. Spectra of intact mutant and normal Hb were recorded on a Cary 2200 spectrophotometer. Hb concentrations are given on the basis of heme.

Mass spectrometric analysis

The purified N102A(β) mutant Hb tetramer was analyzed on a matrix-assisted laser desorption time-of-flight mass spectrometer constructed at Rockefeller University (Beavis & Chait, 1989, 1990). A small aliquot of the sample was applied to the top of probe, dried in air, and then inserted into the mass spectrometer for analysis. In this system, the Hb dissociates into its constituent α- and β-globin chains and heme is released. Horse heart myoglobin was the calibrant.

Determination of amount of Hb dimer

The binding to haptoglobin of dimer dissociated from the N102A mutant tetramer (132 kDa for the complex) and its separation from the unbound Hb tetramer (64 kDa) by use of a Centricon 100 filter was performed as described previously. In some experiments, quenching of fluorescence was used by procedures described previously (Yanase et al., 1994).

Determination of oxygen binding curves

For the recombinant and natural Hb (10 μL, 2.4 mM in heme in 50 mM bis-Tris acetate, pH 7.5), the oxygen binding curves were measured at 37 °C on a modified Hem-O-Scan instrument (Manning, 1981; Yanase et al., 1994). The oxygen equilibrium of lower concentrations of Hb cannot be determined with this instrument. Prior to the analysis, the CO form of Hb, the ligand state in which the mutant Hb was purified to ensure that the heme was not oxidized, was converted to the oxy form by several exposures to incandescent light in an atmosphere of 100% O_2, as described previously (Manning, 1981). This conversion was considered complete when the A_{540}/A_{560} ratio was 1.7. The oxygen equilibrium curves of the recombinant and normal Hb were measured in the absence or presence of 0.5 M chloride. The gas mixture used to achieve oxygenation was either 25% O_2/75% N_2, or 50% O_2/50% N_2 (Medgas).

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Properties of a recombinant human hemoglobin with aspartic acid 99(β), an important intersubunit contact site, substituted by lysine

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Abstract

Site-directed mutagenesis of an important subunit contact site, Asp-99(β), by a Lys residue (D99K(β)) was proven by sequencing the entire β-globin gene and the mutant tryptic peptide. Oxygen equilibrium curves of the mutant hemoglobin (Hb) (2–15 mM in heme) indicated that it had an increased oxygen affinity and a lowered but significant amount of cooperativity compared to native HbA. However, in contrast to normal HbA, oxygen binding of the recombinant mutant Hb was only marginally affected by the allosteric regulators 2,3-diphosphoglycerate or inositol hexaphosphate and was not at all responsive to chloride. The efficiency of oxygen binding by HbA in the presence of allosteric regulators was limited by the mutant Hb. At concentrations of 0.2 mM or lower in heme, the mutant D99K(β) Hb was predominantly a dimer as demonstrated by gel filtration, haptoglobin binding, fluorescence quenching, and light scattering. The purified dimeric recombinant Hb mutant exists in 2 forms that are separable on isoelectric focusing by about 0.1 pH unit, in contrast to tetrameric hemoglobin, which shows 1 band. These mutant forms, which were present in a ratio of 60:40, had the same masses for their heme and globin moieties as determined by mass spectrometry. The elution positions of the α- and β-globin subunits on HPLC were identical. Circular dichroism studies showed that one form of the mutant Hb had a negative ellipticity at 410 nm and the other had positive ellipticity at this wavelength. The findings suggest that the 2 D99K(β) recombinant mutant forms have differences in their heme-protein environments.

Keywords: cooperativity; hemoglobin; hemoglobin intersubunit contact; mass spectrometry; mutagenesis

There are several amino acid side chains at the $\alpha_1\beta_2$ subunit contact of hemoglobin A that are very important in the allosteric transition between oxy- and deoxyhemoglobin (Perutz & Ten Eyck, 1971; Perutz, 1990). One of these is Asp-99(β), which is part of an important segment extending from amino acids 94 through 99 at the FG corner of the β-subunit, a region where the largest structural subunit rearrangements occur in the transition between oxy- and deoxyhemoglobin. In this segment the side chains make contacts with some of the opposite side chains of amino acids 36–44 of the C-helix of the α-chain. The major bonding in which Asp-99(β) participates is with Tyr-42(α) (Kinemage 1). Information on the critical nature of Asp-99(β) came originally from studies with naturally occurring mutant Hb in which the negatively charged side chain was substituted by a variety of other amino acids. These mutant Hb include Hb Kempsey (Asp → Asn) (Reed et al., 1968; Bunn et al., 1974), Hb Yakima (Asp → His) (Jones et al., 1967), Hb Radcliffe (Asp → Ala) (Weatherall et al., 1977), Hb Ypsilanti (Asp → Tyr) (Rucknagel et al., 1967), Hb Hotel-Dieu (Asp → Gly) (Blouquit et al., 1981), Hb Chemilly (Asp → Val) (Rochette et al., 1984), Hb Coimbra (Tamagnini et al., 1991), and Hb Ingelheim (Wajcman et al., 1991) (the latter two have Asp → Glu substitutions). All of these natural mutant Hb exhibit an increased oxygen affinity and a decreased cooperativity. Because none of these has a strongly basic amino acid side chain, we decided to substitute a Lys at this site because such a substitution could have profound effects on subunit interactions at the $\alpha_1\beta_2$ interface.

In order to achieve this objective, we used a yeast expression system in which the genes of the α- and β-chains of human globin are expressed on the same plasmid (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b). This expression system

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Abbreviations: Hb, hemoglobin; 2,3-DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; TFA, trifluoroacetic acid; r, recombinant; PTH, phenylthiohydantoin; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

does not involve manipulations with fusion proteins; it utilizes the endogenous heme of yeast and produces a soluble Hb that is properly folded and processed at its N-terminal amino acids in the same manner as human HbA. This conclusion is based on comparison of the properties of the recombinant Hb with natural human Hb. In the case of sickle Hb, the criteria were mass spectrometry, amino acid analysis, peptide mapping, HPLC, N-terminal protein analysis, C-terminal protein analysis, reactivity of the Cys-93 SH group, spectral properties, oxygen affinity, cooperativity, response to allosteric regulators, and aggregation of the protein (Martin de Llano et al., 1993a, 1993b). Hence, this system was considered ideal to introduce the desired positively charged substitution at the β99 site. This communication describes some unusual properties of this recombinant mutant, i.e., its virtual lack of response to allosteric regulators and its existence in 2 forms.

Results

Purification of the D99K(β) Hb mutant

Yeast harboring the normal human α-globin gene and the β-globin gene mutated to translate for Lys instead of Asp at position 99 did not affect the growth of the yeast. After breakage of the cells and initial batchwise purification on CM-52, the final purification of the D99K(β) recombinant mutant was achieved on HPLC (Fig. 1). The gradient described in the Materials and methods for the purification of the mutant Hb was chosen to ensure the complete removal of several minor components. The major Hb component, which eluted as a symmetrical peak with the main fractions from 34.5 min to 37.5 min, was used for all the structural and functional studies described below.

Characterization of the D99K(β) recombinant mutant Hb

The purified mutant recombinant Hb was subjected to SDS-PAGE, which showed 2 very close and equally intense bands with molecular weights of about 16,000 corresponding to the α- and β-globin chains of Hb (Fig. 2). Analysis of the purified mutant Hb in a nondenaturing electrophoresis system (Beckman Paragon) showed a single band, which moved more toward the cathode than did HbA (data not shown), consistent with the substitution of a negative charge by a positive charge in the mutant Hb β-chain.

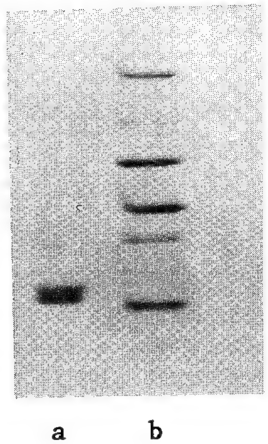

Fig. 2. SDS-PAGE of purified D99K(β) mutant Hb. Lane a, 3 μg of purified mutant Hb was put onto the gel; lane b, marker proteins were bovine serum albumin (66 kDa), egg white albumin (45 kDa), pepsin (34 kDa), trypsinogen (24 kDa), β-lactalbumin (18.4 kDa), and lysozyme (14.3 kDa). An 18% crosslinked gel was used.

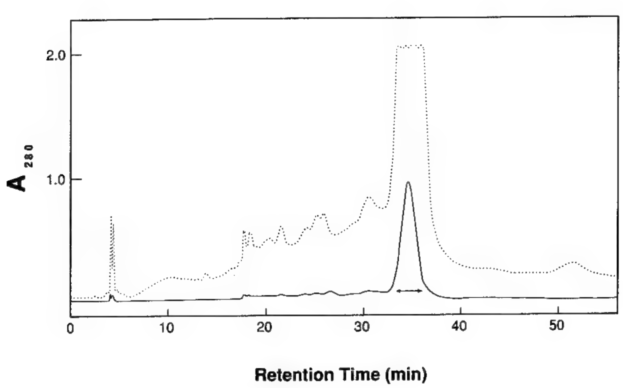

Fig. 1. Purification of D99K(β) mutant Hb by HPLC. The Hb (18 mg) obtained from the yeast extract prepared by chromatography on CM-52 was applied to a SynChropak CM300 column as described in the text. The solid line indicates the absorbance at 280 nm, and the dotted line shows 10 times that absorbance.

HPLC separation as described in the Materials and methods gave 1 α- and 1 β-globin chain. The latter eluted earlier than the normal β-chain; the α-chains from the mutant and normal Hb eluted in practically the same position, consistent with the absence of a substitution in the α-chains. Amino acid analysis of the isolated chains was used for their identification (Table 1). For those amino acids for which there are significant differences between the α- and β-chains, i.e., serine, glutamic acid, glycine, alanine, and valine, there was good agreement between the theoretical values and those actually found; the absence of isoleucine was consistent with the known amino acid sequence of human HbA and the mutant recombinant Hb.

Peptide mapping and sequencing

In order to confirm the substitution site, about 300 μg of each normal and mutant β-chain were digested with TPCK-trypsin and the digests were subjected to HPLC analysis. The pattern of the tryptic peptide map of mutant β-globin chain was compared to the corresponding tryptic map of the normal β-globin chain (Fig. 3). A major peptide from the mutant chain at 40 min retention (bottom panel, open arrow) replaced 1 tryptic peptide from normal HbA (top panel, closed arrow); the rest of both peptide maps had the same profile, consistent with a single substitution on the protein.

The peptide from the mutant Hb (bottom panel) was subjected to sequencing by Edman degradation. The results, shown in Figure 4, indicated a sequence corresponding to that of amino acids 83–102; the underlined Lys was the site of the substitution. The high yield of the PTH-Lys residue at position 99 confirmed that the mutant β-globin chain contained the single amino acid substitution β99Asp $\rightarrow$ Lys. In this isolated tryptic peptide, there were 2 Lys residues that were not cleaved by trypsin: the Lys^{95}–

Table 1. *Amino acid analysis of α- and β-chains of recombinant D99K(β)*[a]

Amino acid	Found α-Chain	Found β-Chain	Theory α-Chain	Theory β-Chain
Lysine	10.4	11.3	11	12
Histidine	10.5	9.5	10	9
Arginine	2.8	2.9	3	3
Aspartic acid	11.3	11.0	12	12
Threonine	7.5	6.0	9	7
Serine	11.1	5.3	11	5
Glutamic acid	5.9	11.5	5	11
Proline	6.7	6.9	7	7
Glycine	7.9	12.6	7	13
Alanine	19.0	15.8	21	15
Valine	12.1	15.3	13	18
Isoleucine	0	0	0	0
Leucine	18.0	18.0	18	18
Tyrosine	1.9	1.3	3	3
Phenylalanine	7.6	6.7	7	8

[a] For the amino acids in bold, there are significant differences between the amounts in the α- and β-chains and these amounts are underlined. The results were compiled from 2 separate analyses. The values for Thr, Ser, Cys, Met, Tyr, and Trp are low or absent because they are partially or completely destroyed during acid hydrolysis. The values for Val are low because of incomplete hydrolysis of Val–Val bonds during the 24-h acid hydrolysis.

Leu[96] bond, which is preceded by Asp[94], and the Lys[99]–Pro[100] bond.

Oxygen binding properties of the D99K(β) mutant Hb

In view of the importance of the Asp-99(β) site in the transition between the oxy and the deoxy states of Hb (Turner et al., 1992), the oxygen binding properties of this mutant were measured (Fig. 5). As reported for the isolated natural Hb that have mutations at this position, the oxygen affinity of the recombinant mutant was increased. However, unlike most of the naturally occurring mutants at this position, the shape of the curve of the D99K(β) mutant Hb indicated that it retained significant cooperativity (Fig. 5, inset; Table 2). Unlike normal HbA, the D99K(β) mutant did not respond to added chloride by undergoing a decrease in its oxygen affinity. Furthermore, it responded only marginally to 2,3-DPG and to added IHP (Table 2).

With the Hem-O-Scan instrument it was not possible to measure the oxygen binding curve of Hb at concentrations lower than about 1.5 mM in heme, but higher concentrations were analyzed to determine any changes in oxygen affinity and cooperativity. Thus, increasing the concentration nearly 8-fold had only a slight effect on the P_{50} (Table 2). The maximum P_{50} value attained with the D99K(β) recombinant mutant was about 11 mm Hg, and this oxygen affinity was not further lowered by addition of chloride.

It is important to note that other recombinant Hb expressed in this system, i.e., HbS, have functional proteins that are fully responsive to chloride (Martin de Llano et al., 1993a) and to 2,3-DPG (Table 2). Hence, the lack of a response to chloride is a property of the D99K(β) mutant.

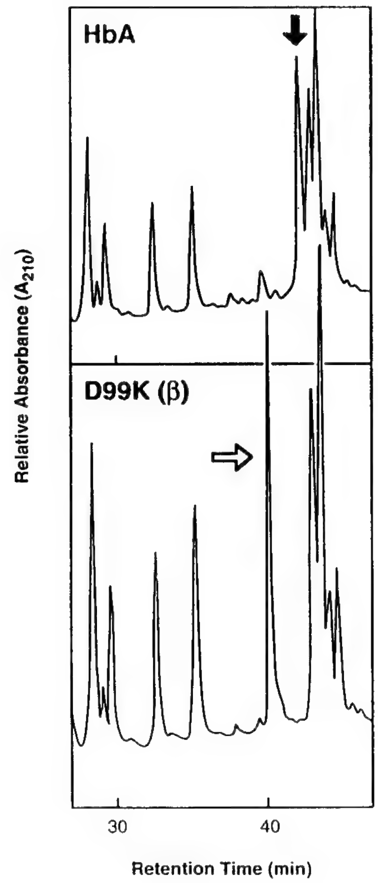

Fig. 3. Tryptic peptide maps of β-chain of D99K(β) mutant Hb and HbA. The only part of the tryptic peptide map shown is the region where the change occurred; the other parts of the maps were identical. The β-chains of each Hb were isolated by HPLC, reduced, carboxymethylated, and digested with TPCK-trypsin, as described in the text. Chromatography was performed on a Vydac C-18 column as described in the text. The closed and the open arrows indicate unique peaks in digests of HbA and D99K(β) mutant, respectively. The peptide in the lower peak (open arrow) from the mutant Hb was subjected to sequencing as described in the text and in Figure 4.

The effect of 2,3-DPG on the oxygen binding of an equimolar mixture of the recombinant mutant Hb and natural HbA was an average of the contribution from each Hb (Table 2). However, this extent of oxygen release was much less than that for HbA in the presence of allosteric regulators. If present in an erythrocyte in such amounts, this mutant Hb would seriously impede the ability of the red cell to release oxygen.

Dissociation of the mutant Hb

The properties of several natural Hb with mutations at Asp-99(β) have been reported in some detail by several investigators (Jones et al., 1967; Rucknagel et al., 1967; Reed et al., 1968; Bunn et al., 1974; Weatherall et al., 1977; Blouquit et al., 1981; Turner et al., 1981, 1992; Rochette et al., 1984; Tamagnini et al., 1991; Wajcman et al., 1991; Doyle et al., 1992). In general, each

Gly[83]Thr[84]Phe[85]Ala[86]Thr[87]Leu[88]Ser[89]Glu[90]Leu[91]His[92]Cys(Cm)[93]Asp[94]Lys[95]Leu[96]His[97]Val[98]Lys[99]Pro[100]Glu[101]Asn[102]

333 134 303 249 111 203 46 162 155 166 - 183 124 122 134 133 108 112 95 87

Fig. 4. Sequence of mutant tryptic peptide from D99K(β). The peptide that was sequenced was isolated as described in Figure 3; the mutant peptide is designated by the open arrow. The values in parentheses are the number of picomoles of each PTH-amino acid found at each cycle of sequencing.

natural mutant showed greater susceptibility toward tetramer–dimer dissociation in both the oxygenated and the deoxygenated states. For example, whereas the dimer–tetramer dissociation constant for normal human HbA is about 10^{-5} M in its oxygenated state and 10^{-10} M in its deoxygenated form (Turner et al., 1981), the corresponding values for the natural mutants at Asp-99(β) are increased by 3–5 orders of magnitude (Turner et al., 1981, 1992; Doyle et al., 1992). We have measured the extent of dimer formation of the oxygenated D99K mutant Hb by several procedures including gel filtration, haptoglobin binding of $\alpha\beta$ dimers determined by filtration of the haptoglobin–Hb dimer complex and by quenching of fluorescence, and by light scattering, as described below.

When the oxygenated D99K(β) mutant Hb (0.4 mM in heme initially) was applied to Sephadex G-75, it eluted in the position corresponding to that of the dimer. Under the same conditions, oxygenated human HbA eluted in a position corresponding to a tetramer.

Light scattering experiments of the oxygenated recombinant D99K(β) mutant Hb showed that, at the Hb concentrations analyzed (0.2 mM in heme), the mutant Hb existed predominantly in the dimeric form (38,000 molecular weight; average of 13 determinations).

Haptoglobin binds Hb dimers instantaneously and very tightly, but tetramers are not bound (Nagel & Gibson, 1967; Benesch et al., 1976). Human HbA undergoes dissociation to dimers more readily in its oxygenated compared to its deoxygenated form (Nagel & Gibson, 1967; Benesch et al., 1976; Doyle et al., 1992; Turner et al., 1992). Like oxy HbA, the D99K(β) mutant Hb in its oxygenated state dissociated readily to form dimers that combined with haptoglobin (Table 3). Deoxy HbA slowly forms dimers that react with haptoglobin (Doyle et al., 1992; Turner et al., 1992). The value of about 20% dimer formation from deoxy HbA during the time period of the experiment in Table 3 was consistent with its slow dissociation. In the deoxygenated state and at the concentration studied, the mutant D99K(β) Hb bound to haptoglobin much more readily than does deoxy HbA (Table 3), indicating that it was mainly dimeric at this concentration.

The quenching of haptoglobin fluorescence by Hb dimers (Nagel & Gibson, 1967) can also be used to determine the presence of dimers because Hb tetramers do not have this property. As shown in Table 3, the quenching of haptoglobin fluorescence by oxy HbA was readily demonstrated. However, at this concentration (4 μM in heme), deoxy HbA was present mainly in the tetrameric state and thus shows very little quenching of fluorescence. In contrast, the extent of quenching of fluorescence of both the oxy and the deoxy D99K(β) mutant Hb was about the same as that of oxy HbA. These results are consistent with those found by the other procedures described above and

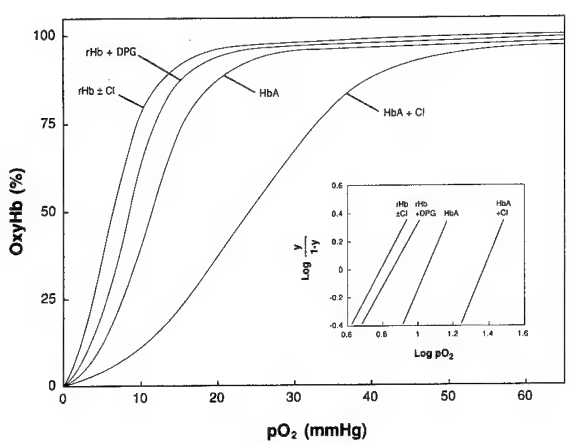

Fig. 5. Oxygen binding curves and Hill plots of D99K(β) recombinant mutant Hb and natural HbA. The oxygen equilibrium curves were determined at 37 °C on Hb (2.0 mM in heme) in 50 mM bis-Tris acetate, pH 7.5. The effect of anions on the oxygen-binding affinity of Hb was determined in the presence of 0.5 M NaCl (Cl) or 1 mM 2,3-DPG as described in the text. Hill plots, the logarithm of the fractional oxygen saturation (y) plotted against the logarithm of the partial pressure of oxygen (pO_2), for each sample are shown in the **inset**.

Table 2. *Effect of anions on the oxygen binding properties of recombinant D99K(β) mutant Hb*[a]

Hemoglobin	Conc (mM)	Addition	P_{50}	n
HbA (natural)	1.6	0	11	2.5
		0.5 M NaCl	24	2.5
HbS (recombinant)	1.2	1 mM 2,3-DPG	25	3.0
D99K(β)	1.6	0	6	2.1
		0.1 M NaCl	7	2.2
		0.5 M NaCl	6	2.1
		1 mM 2,3-DPG	8	2.3
		1 mM IHP	9	2.2
D99K(β)	0.8	0	10	2.5
+				
HbA	0.8	1 mM 2,3-DPG	15	2.7
D99K(β)	3.7	0	8	2.4
D99K(β)	7.3	0	10	ND[b]
		0.1 M NaCl	9	2.5
D99K(β)	14.6	0	11	2.5

[a] The P_{50} values, which are averages of 5 determinations, are expressed in mm Hg and have a precision of ±1 mm Hg. The Hb concentrations are given as heme values.

[b] ND, not determined.

Table 3. *Haptoglobin binding to normal HbA and recombinant Hb mutant D99K(β)*

Hb sample	% Dimer by filtration[a]	% Fluorescence quenching[b]
Oxy HbA	69	55
Deoxy HbA	19	9
Oxy D99K(β)	72	52
Deoxy D99K(β)	71	53

[a] In the filtration assay, the concentrations of HbA and the D99K(β) mutant were 2×10^{-6} M in heme. A Centricon 100 was used to determine the amount of dimer bound to haptoglobin, as described in the text. The amount of Hb–haptoglobin complex retained by the filter divided by the total amount of Hb is expressed as the % dimer.

[b] In the fluorescence assay, the concentrations of Hb and haptoglobin were 1×10^{-6} M each and the extent of quenching was determined as described in the text.

showed that the mutant was nearly completely dimeric at the concentrations studied.

The data above are to be taken only as an indication of the ease of dimer formation for the mutant Hb and are not meant to indicate that it is dimeric at all concentrations. The measurements, which were performed at Hb concentrations of 0.2 mM in heme or lower, indicated that the mutant Hb in either the oxy or the deoxy state was dimeric at these concentrations. Indeed, we could not detect any tetrameric mutant Hb by any of the 4 methods described above. However, these procedures could not be used at high Hb concentrations. Further studies using modified or novel procedures will be needed to measure any tetramer–dimer dissociation constant of this recombinant Hb with its strongly altered intersubunit contact.

Isoelectric focusing

Analysis of the recombinant D99K(β) mutant by isoelectric focusing showed the presence of 2 somewhat diffuse bands with p*I* values that differ by 0.1 pH unit (Fig. 6A, lane b). Also shown are the electrophoretic behavior of purified natural HbA from normal red cells (lane c) and recombinant sickle Hb (lane a) from the yeast expression system. The bands from these latter 2 tetrameric Hb were not diffuse like the 2 bands from the D99K(β) Hb. Thus, the doublet pattern of the D99K(β) mutant is not typical of Hb expressed in the yeast system. These bands are referred to as forms D99K(β)-1 and D99K(β)-2.

The 2 forms of the D99K(β) mutant Hb were shown not to be due to oxidation because the spectra of the isolated D99K(β)-1 and D99K(β)-2 (see below) indicated that the amount of met-Hb present, if any, was less than 5%. Second, deliberate oxidation of the D99K(β) mutant with potassium ferricyanide prior to isoelectric focusing resulted in slightly different mobilities of the 2 bands but did not change their relative amounts.

Distribution of the two forms of the D99K recombinant mutant

After isoelectric focusing of different amounts of Hb, the relative intensities of the 2 stained bands were determined by scanning the stained bands on the gel, as described in the Materials and methods. The relative amounts of each were about 40% for the D99K(β)-1 band and about 60% for the D99K(β)-2 band (Table 4).

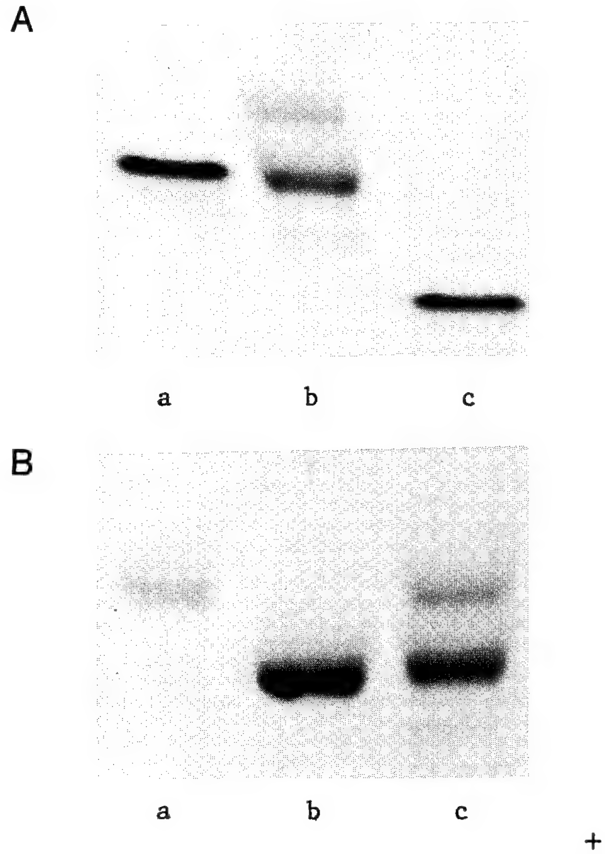

Fig. 6. Isoelectric focusing of D99K(β) mutant Hb. A gel (pH 6–8) (Isolab) was electrophoresed at 10 W for 40 min and stained with 0.2% bromophenol blue. **A:** Lane a, recombinant HbS; lane b, recombinant D99K; lane c, natural HbA. **B:** The separated D99K(β)-1 and D99K(β)-2 bands were eluted from the isoelectric focusing gel as described in the text and then reapplied to a new gel and subjected to isoelectric focusing. Lane a, D99K(β)-1 (60 μg); lane b, D99K(β)-2 (100 μg); lane c, fresh D99K(β) (141 μg).

Separation and properties of the 2 forms of the D99K mutant Hb

After isoelectric focusing, the unstained D99K(β)-1 and D99K(β)-2 forms, which were located by their red color, were eluted from the gel with CO-saturated 50 mM bis-Tris acetate, pH 7.5. They were recovered with a yield of 75% in the approximate ratio of 40:60. A second isoelectric focusing of each separated Hb showed that there was no interconversion of the 2 Hb forms during the electrophoresis (Fig. 6B). Furthermore, the D99K(β)-1 and D99K(β)-2 forms were not formed as a result of the electrophoresis.

The spectral properties of the CO derivatives of the D99K(β) mutant Hb before isoelectric focusing and of the 2 forms separated during isoelectric focusing are given in Table 5. The cor-

Table 4. *Distribution of two D99K(β) forms*[a]

Hb concentration applied (μM)	D99K(β)-2 (%)	D99K(β)-1 (%)
100	62	39
500	62	39
1,000	65	36

[a] The actual Hb concentration during the electrophoresis may be different from the amount applied. After isoelectric focusing, each band was eluted from the gel and the amount of each was determined by densitometry as described in the text.

responding values for HbA are also given for comparison. The results show that the spectral properties of all samples are practically the same at each of the major wavelengths and indicate the absence of met-Hb.

Isolation of the globin chains from major and minor forms

The characterization and sequencing of the mutant tryptic peptide described above were fully consistent with the desired mutation. However, in order to exclude the possibility of an alteration in some other part of the protein chain that could be responsible for the 2 forms of the D99K(β) mutant Hb, the α- and β-globin chains from D99K(β)-1 and D99K(β)-2 were analyzed. HPLC analysis of the unfractionated D99K(β) Hb before isoelectric focusing showed that its β-chain eluted at 18.2 min and the α-chain eluted at 27.4 min (Fig. 7, top panel); the reproducibility was ±1 min on the HPLC. HPLC analysis of the separated D99K(β)-1 and D99K(β)-2 showed that their β-chains eluted at 18.8 and 18.2 min, respectively, and the α-chains at 28.6 and 27.6 min, respectively (Fig. 7, second and third panels). These values are identical within experimental error. By comparison, HPLC analysis of HbA (Fig. 7, bottom panel) showed that the normal β-chain eluted at 21.3 min (average of 2 determinations, ±0.9 min) and the α-chain eluted at 27.9 min (average of 2 determinations, ±1.5 min). Therefore, differences between the 2 components due to primary sequence or posttranslational changes were not evident by these analyses.

Table 5. *Spectral properties of D99K(β) isoelectric focusing forms*[a]

	Spectral ratio	
Sample	272/539	420/539
HbA	2.64	14.4
D99K(β) (before isoelectric focusing)	2.69	14.8
D99K(β) (extracted)	2.67	14.5
D99K(β)-1	3.02	14.9
D99K(β)-2	2.70	14.4

[a] The Hb are the CO derivatives. At absorbance values of 4.0 or less, the precision and accuracy of the values are within ±1%.

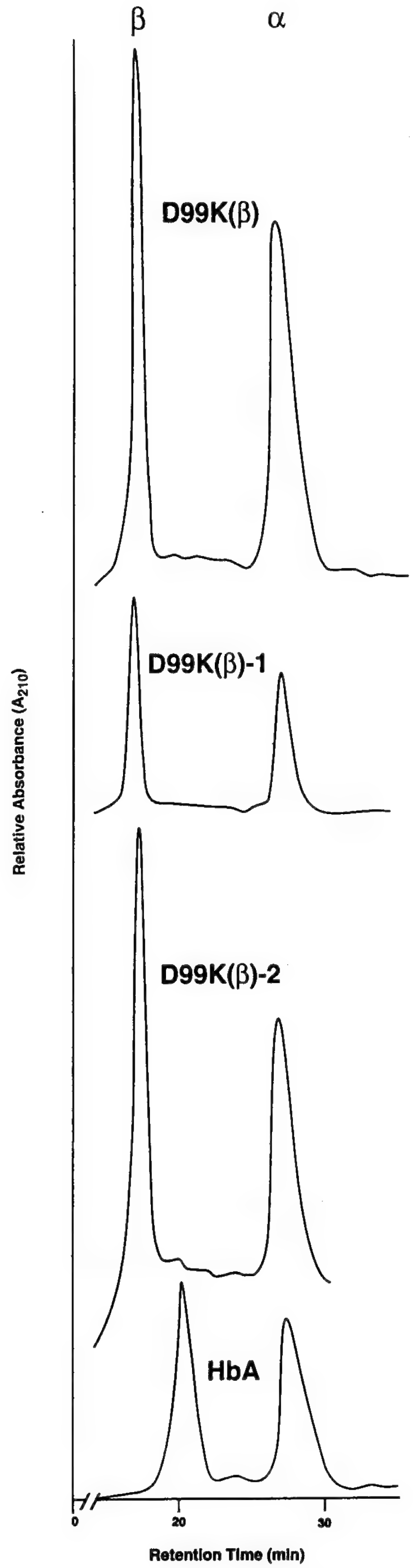

Fig. 7. Separation of α- and β-globin chains of D99K(β) mutant Hb. The D99K(β) and isolated D99K(β)-1 and 2 were chromatographed on a Vydac C-4 column as described in the text. The amounts of hemoglobin applied were in the range of 100–200 μg.

Table 6. *Mass spectrometric analysis of the 2 forms of the D99K(β) Hb mutant*[a]

	Mass					
	Heme		α-Globin		β-Globin	
Hb form	Measured	Calculated	Measured	Calculated	Measured	Calculated
D99K(β)-1	616.8	616.5	15,127.1	15,126.4	15,881.4	15,881.3
D99K(β)-2	616.6	616.5	15,124.4	15,126.4	15,879.6	15,881.3

[a] The analyses for heme and globin masses were performed as described in the text. The precision for the globin measurements is ±2.0 Da and for the heme measurements ±0.5 Da.

The possibility that the different mobilities of D99K(β)-1 and D99K(β)-2 could be due to heme was evaluated below.

Mass spectrometric analysis of separated D99K(β)-1 and D99K(β)-2

In order to investigate the possibility that some subtle change (not detectable by HPLC analysis) could have occurred on the protein, the separated D99K(β)-1 and D99K(β)-2 were each subjected to matrix-assisted laser desorption mass spectrometric analysis. The results, which are shown in Table 6, indicate that the α- and β-subunits of D99K(β)-1 and D99K(β)-2 have identical masses within the error of the measurements (±2 Da). The measured increase in mass of the mutant D99K(β)-1 and D99K(β)-2 chains over a normal β-chain were, respectively, 13.2 ± 2 Da and 11.4 ± 2 Da, values consistent with the increase of 13 Da calculated for the replacement of 1 aspartic acid by a lysine residue. The identical molecular masses of the 2 forms are consistent with identical primary structures and also indicate that modification during isoelectric focusing did not occur.

The possibility that yeast contains different types of heme moieties that could lead to the generation of the 2 D99K(β) recombinant mutant forms was evaluated by matrix-assisted laser desorption mass spectrometric analysis. The results, which are shown in Table 6, indicate that the heme moieties derived from D99K(β)-1 and D99K(β)-2 have masses that are identical within the accuracy of the measurement (±0.5 Da), demonstrating that such a difference in the heme moieties is unlikely. Because the mass spectrometric results show that the primary sequence and the heme prosthetic group in the 2 forms of the D99K(β) recombinant mutant Hb are the same, the possibility was considered that there was some type of conformational difference between the 2 forms of the D99K(β) recombinant mutant as described next.

Circular dichroism spectra

The circular dichroic spectrum in the 200–300-nm range of the D99K(β) mutant prior to isoelectric focusing and the corresponding spectra of the 2 separate D99K(β)-1 and D99K(β)-2 indicated that the differences were insignificant (data not shown). However, in the Soret region at 410 nm, D99K(β)-1 displayed negative ellipticity, a feature absent in D99K(β)-2 (Fig. 8). The overall circular dichroism spectrum and positive ellipticity of D99K(β)-2 in the Soret region were practically identical to that of HbA. These results suggested that the heme-protein environment of D99K(β)-2 was closer to that of natural HbA than that of D99K(β)-1. In addition, the degree of ellipticity at 410 nm for the D99K(β) recombinant mutant as well as that at 345 nm before isoelectric focusing reflected the contributions of D99K(β)-1 and D99K(β)-2 at each wavelength. These observations are not consistent with artifact formation of these bands during isoelectric focusing, but the relationship between their electrophoretic behavior and circular dichroism properties is not known. In a separate study using a different sample of the D99K(β) Hb mutant from a different yeast preparation, circular dichroism results similar to those described in Figure 8 on the separated D99K(β)-1 and D99K(β)-2 bands were obtained.

Discussion

There are 8 known natural Hb mutants with substitutions at Asp-99(β) (Jones et al., 1967; Rucknagel et al., 1967; Reed et al., 1968; Bunn et al., 1974; Weatherall et al., 1977; Rochette et al., 1984; Tamagnini et al., 1991; Wajcman et al., 1991). In the absence of allosteric regulators, the oxygen binding curves of these natural mutants in dilute solution do not show cooperativity (n values near 1) and they have an increased oxygen affinity. Both properties have been attributed to the increased dissociation of the tetrameric state of these natural mutant Hb into dimers. As

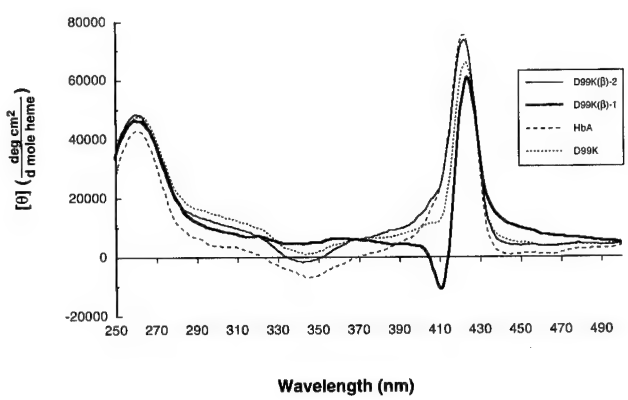

Fig. 8. Circular dichroism spectra of D99K(β) mutant Hb. The spectra were recorded at the concentration of approximately 30 μM mutant Hb as the CO derivative and have been corrected for small differences in protein concentration. The D99K(β)-1 and D99K(β)-2 forms were isolated after isoelectric focusing, as described in the text. The measurements were made on unstained bands from the gel.

described in this communication, the D99K(β) mutant has a reduced but not absent cooperativity ($n = 2$) compared to that of normal Hb ($n = 3$). Our results using haptoglobin binding, light scattering, fluorescence quenching, and gel filtration indicate predominantly a dimeric structure for the D99K(β) mutant in either the oxygenated or deoxygenated state at concentrations in the range of 0.2 mM or less in heme. However, at the Hb concentration used for measurement of the oxygen dissociation curves (2–15 mM in heme), it is not known how much of the mutant Hb is tetrameric or dimeric because the 4 methods used above to measure the tetramer–dimer dissociation cannot be used at such high Hb concentrations. Existing procedures must be modified or novel ones devised for these measurements. However, the absence of large changes in oxygen affinity over the 8-fold change in Hb concentration suggests a preponderance of the dimeric structure.

Of particular interest is the lack of an effect of chloride and the marginal effect of the allosteric regulators 2,3-DPG or IHP on the oxygen equilibrium curve of the recombinant mutant Hb D99K(β) compared to HbA. This result may be due to the presence of dimeric Hb because a major mechanism by which chloride leads to a decreased oxygen affinity is by neutralizing positive charges in the 2,3-DPG cleft and in the central dyad axis (Chiancone et al., 1975; Nigen & Manning, 1975; Bonaventura et al., 1976; Bonaventura & Bonaventura, 1978; Manning et al., 1978; Nigen et al., 1980; Fronticelli et al., 1988; Vandegriff et al., 1989; Ueno & Manning, 1992; Perutz et al., 1993; Ueno et al., 1993). Both of these anion binding regions are hallmarks of the tetramer but are absent in the dimer. The importance of the central dyad axis in the control of oxygen affinity by chloride has recently been shown by structural studies comparing human and bovine Hb (Perutz et al., 1993) and chemical modification studies showing that both Hb had related functional chloride binding sites (Ueno & Manning, 1992; Ueno et al., 1993). Therefore, the lack of a chloride response in lowering the oxygen affinity is consistent with the absence of these quaternary features in the mutant Hb at the concentration studied. The marginal response to 2,3-DPG and IHP could be due either to the presence of some tetrameric Hb or to its generation in the presence of these effectors; further study is needed to clarify this point. The data indicate that the presence of equivalent amounts of the mutant Hb and HbA in a heterozygous erythrocyte would lead to a significant reduction in the amount of oxygen released even in the presence of allosteric regulators. Therefore, under physiological conditions, this mutant Hb in a red cell would likely be dysfunctional.

It is informative to compare the properties of the D99K(β) Hb mutant with the 2 recombinant mutants of Tyr-42(α), the site closely linked to Asp-99(β) at the $\alpha_1\beta_2$ subunit contact in deoxy Hb (see Kinemage 1). Imai et al. (1991) reported that recombinant mutants with phenylalanine or histidine substitutions at this position, Y42F(α) and Y42H(α), respectively, had an increased oxygen affinity and a decreased cooperativity, properties shared with the D99K(β) mutant. Of possible relevance to the findings in the present communication is the observation that the Y42H(α) mutant had an n value of 2 at pH 6.8, a value similar to that for D99K(β) reported here. At higher pH there was diminished yet measurable cooperativity for the Y42H(α) mutant. Imai et al. (1991) attributed the mild functional loss in the Y42H(α) mutant to the presence of a weak hydrogen bond formed in the deoxy state between His-42(α) and Asp-99(β) in the mutant Hb. Whether there is any type of bonding between the protonated ϵ-NH_2 group of the mutant Lys-99(β) and any other side chains must await the solution of the crystal structure of the D99K(β) Hb mutant forms, which may also shed light on the source of its cooperativity. Vallone et al. (1993) have demonstrated the importance of other α-chain subunit contacts in the general region of the $\alpha_1\beta_2$ contact.

It is also possible that the introduction of a Lys residue has created a new chloride binding site, analogous to that recently reported by Rivetti et al. (1993) for Hb Rothschild (Trp-37(β) → Arg). Studies on other recombinant Hb (Baudin et al., 1993) with substitutions in this region of the protein not found in natural Hb mutants will likely continue to reveal information on this important region of the Hb molecule.

Because of the choice of substitution site, the D99K(β) recombinant mutant shows a tendency to dimerize, whereas other recombinant Hb expressed in this expression system remain tetrameric under similar conditions. Indeed, most mammalian Hb are tetrameric, enabling them to interact efficiently with allosteric regulators. However, some dimeric Hb in the unliganded form, i.e., from the mollusc bivalve clams, show very similar negative and positive ellipticity in the region of the Soret band as found for the D99K(β)-1 Hb (Chiancone et al., 1981, 1990; Antonini et al., 1984; Bellelli et al., 1987). The interaction of the heme with 2 different environments of the adjacent globin may be responsible for generation of the D99K(β)-1 and D99K(β)-2 forms. Alternatively, the 2 forms could be related to the reports of a "disordered" heme environment in some monomeric Hb and in some myoglobins. Thus, La Mar and colleagues (Burns & La Mar, 1981), using NMR techniques, showed that the heme of tuna myoglobin has 2 different orientations present in a 60:40 ratio, similar to the distribution of the 2 forms of the D99K(β) mutant found in the present study. Cooke and Wright (1985) also reported a difference in heme orientation in the monomeric Hb of *Glycera*, and Constantinidis and Satterlee (1987) found that these forms were separable by isoelectric focusing. O'Connor et al. (1980) and Santucci et al. (1988) reported a correlation between heme orientation and circular dichroism in the Soret region of *Glycera* Hb. In their description of heme disorder in myoglobin, Light et al. (1987) showed a correlation between heme orientation and the degree of circular dichroic ellipticity in myoglobin. Of particular interest is the observation of Goodhall and Shooter (1969) that the negative ellipticity in the isolated β-chain of HbA in the Soret region is lost when tetrameric Hb is formed. On the other hand, there could be a protein conformational change that gives rise to the 2 forms of the D99K(β) mutant.

Other heme proteins that are homogeneous by other criteria also show 2 major bands on isoelectric focusing. Thus, Hull and Wharton (1993) showed that cytochrome oxidase/nitrite reductase, a protein shown to be pure by several criteria, had 2 bands separable by about 0.05–0.10 pH unit on isoelectric focusing. These 2 forms were interconvertible upon a subsequent isoelectric focusing, but the D99K(β) mutant Hb forms were not under such conditions. This difference could be a reflection of the homodimeric nature of cytochrome oxidase/nitrite reductase compared with the heterodimeric structure of the D99K(β) recombinant mutant at the concentrations used for the isoelectric focusing studies. Further studies are needed to elucidate the molecular basis for the existence of 2 forms of the mutant Hb and their relationship, if any, to the cooperativity of the mutant Hb.

Materials and methods

Reagents

The restriction endonucleases and other enzymes were from Boehringer Mannheim. The DNA sequencing kit and the T7 DNA polymerase (Sequenase, version 2.0) were obtained from U.S. Biochemicals. The oligonucleotide used to make the mutation had the sequence [5′-CTGAAGTTCTCAGGTTTCACG TGCAGCTTG-3′] and was purchased from Operon Technologies (Alameda, California). The underlined bases were used to produce the desired mutant.

Bacterial and yeast strains and growth conditions

These strains have been described previously (Martin de Llano et al., 1993b). The yeast strains were grown in 10 culture flasks (2 L each) for 4 days in the presence of ethanol as the carbon source, and Hb expression was induced by the addition of 2% galactose for 24 h as described previously (Martin de Llano et al., 1993a, 1993b).

Site-directed mutagenesis

The plasmid pGS189 and pGS389 that contained the full-length human α- and β-globin cDNAs under transcriptional control of dual pGGAP promoters were used as gene sources. The 1.2-kb *Sph* I fragment containing the β-globin cDNA on pGS189 was inserted into the *Sph* I site on the replicative form of M13mp18, so that the phage contains the sense DNA strand of the β-globin gene. *Escherichia coli* BW 313 was transfected with the recombinant phage and the oligonucleotide described above was used to create the mutation β99Asp → Lys by the method of Kunkel (1985).

The presence of the mutation was screened by loss of the *Bam* HI site, which cleaves at the GGATCC sites marked by asterisks:

GTG* GAT C*CT → GTG TTT CCT

Val Asp Pro → Val Lys Pro

Sequencing of the entire β-globin gene indicated that this was the only site of mutation. The mutated β-globin region was excised by *Sph* I digestion and subcloned back into the *Sph* I site of pGS189. The correct orientation of the recombinant fragment was confirmed by DNA sequencing. The plasmid pGS18999K was treated with *Not* I to release the cassette with the mutated globin gene, which was subsequently inserted into the expression vector pGS389 that had been digested with the same restriction enzyme. The correct orientation of the DNA fragment was again confirmed by DNA sequencing, and the recombinant plasmid was transformed into yeast GSY112 *cir*^{0} strain. Transformants were selected on a complete minimal agar plate without uracil.

Protein purification

Upon completion of growth, the yeast cells were saturated with CO gas, collected, broken by homogenization in a Bead-Beater, as described previously (Martin de Llano et al., 1993a, 1993b). The initial purification step was achieved on carboxymethylcellulose (Whatmann, CM-52) and the final purification on HPLC employed the same Synchropak CM 300 (250 × 10 mm) column used previously (Martin de Llano et al., 1993a, 1993b). However, with this mutant Hb, a different gradient was employed because of its more basic properties compared with HbA. The gradient, which consisted of 10 mM potassium phosphate buffer, pH 5.85, and 22.5 mM potassium phosphate buffer, pH 8.0 (150 mL of each), was from 20% to 85% buffer B over 10 min, from 85% to 100% buffer B over 60 min, and then 20 min of buffer B alone at a flow of 2.5 mL/min (A = 30 mM bis-Tris, 30 mM sodium acetate, 1 mM EDTA, pH 6.4; B = 30 mM bis-Tris, 150 mM sodium acetate, 1 mM EDTA, pH 6.4).

Analytical procedures

SDS-PAGE was performed on the recombinant D99K(β) mutant, as described previously (Martin de Llano et al., 1993b). Electrophoresis of native proteins was done on the Beckman Paragon system. Globin chains were separated by HPLC on a Vydac C-4 column (250 × 4.6 mm) using a gradient from 0.1% TFA to 80% acetonitrile in 0.1% TFA. Amino acid analysis of globin chains isolated by this procedure was performed on a Beckman 6300 instrument with System Gold data handling system. Tryptic peptide mapping was performed on the reduced, carboxymethylated globin chains, as described previously (Martin de Llano et al., 1993a). However, the gradient used for separation of the tryptic peptides of the mutant β-chain was changed to 0% to 15% B over 10 min, 15% to 55% B over 60 min, and then 55% to 100% B over 10 min (A = 0.1% TFA; B = 80% acetonitrile, 0.1% TFA). Sequencing of the isolated peptide was performed on an Applied Biosystem gas-phase sequencer. Spectra were recorded on a Cary 2200 spectrophotometer.

Mass spectrometry analysis

Hb samples were subjected to mass spectrometric analysis on a matrix-assisted laser desorption time-of-flight mass spectrometer constructed at The Rockefeller University and described elsewhere (Beavis & Chait, 1989, 1990). The mass spectra were acquired by adding the individual spectra of 200 laser shots. Hb samples isolated from the isoelectric focusing gels but not stained with a dye were prepared for laser desorption mass analysis as follows: the laser desorption matrix material (4-hydroxy-α-cyano-cinnamic acid) was dissolved in formic acid/water/isopropanol 1:6:4 (v/v/v) (50 mM). A 10 mM potassium phosphate solution (pH 8) containing the Hb sample was then added to the matrix solution to give a final concentration of the Hb of approximately 2 μM. A small aliquot (0.5 μL) of this mixture was applied to the metal probe tip and dried at room temperature with forced air. The sample was then inserted into the mass spectrometer and analyzed. Horse heart myoglobin and, after confirmation of its molecular weight, Hb α-chain were used to calibrate the mass spectra.

Determination of tetrameric and dimeric Hb

By binding to haptoglobin

Separation of the Hb tetramer (64 kDa) from the haptoglobin-bound dimer (132 kDa) was achieved by use of a Centricon 100 filter. Haptoglobin-bound dimer remained in the retentate, whereas free Hb tetramer passed through the membrane. The

concentration of the Hb was determined spectrophotometrically before and after the filtration process. Haptoglobin (1 μM) was mixed with oxy Hb or deoxy Hb (2 μM in heme) in 0.1 M potassium phosphate, pH 7.0, at ambient temperature. The mixture was then filtered through a Centricon 100 concentrator (Amicon) by centrifugation at 1,000 $\times g$ for 10 min; concentrations of 50 μM Hb or higher could not be filtered. For deoxy Hb, anaerobic conditions were achieved by addition of 9 mM sodium dithionite to both the Hb and the haptoglobin solutions before they were mixed. After filtration through the Centricon 100, the spectrum of the deoxy Hb sample showed that the Hb remained deoxygenated throughout the filtration process.

By quenching of haptoglobin fluorescence

Equivalent concentrations of oxy and deoxy Hb (2.0–8.0 μM in heme) were mixed with 1 μM haptoglobin, and the decrease in fluorescence (Nagel & Gibson, 1967) was measured on a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer using an excitation wave of 287 nm and an emission wave of 350 nm. Anaerobic conditions were achieved by bubbling the Hb solution in a sealed cuvette with water-saturated N_2 for 2 min prior to addition of dithionite (0.20 mM) and a haptoglobin solution containing 0.20 mM dithionite. After incubation for ~2 min, the fluorescence value was determined. The spectra of the deoxy samples indicated that the Hb had remained in the deoxy state during the fluorescence measurement.

By gel filtration

This procedure was only performed with oxygenated Hb. A column of Sephadex G-75 (0.9 $\times$ 60 cm) in 50 mM bis-Tris acetate, pH 7.5, was eluted at a flow rate of 0.2 mL/min. The Hb samples were applied in a volume of about 100 μL.

By light scattering

Solutions of oxygenated HbA or D99K(β) mutant (ca. 2 mL, 0.2 mM in heme) in 0.1 M KCl were analyzed on a Biotage molecular size detector (model dp-801).

Determination of oxygen binding curves

For the recombinant and natural Hb (ca. 2–15 mM [heme] of each in 50 mM bis-Tris acetate, pH 7.5 at 37 °C), the oxygen binding curves were measured on a modified Hem-O-Scan (Martin de Llano et al., 1993a); the tank containing the oxygenating gas had 25% O_2. Just prior to this analysis, the CO form of Hb, the ligand state in which the mutant Hb was purified to ensure that the heme was not oxidized, was converted to the oxy form by several exposures to incandescent light in an atmosphere of 100% O_2, as described previously (Manning, 1981). This conversion was considered complete when the A_{540}/A_{560} ratio was 1.7.

To measure the effect of anions on the oxygen affinity of the D99K(β) mutant Hb, an aliquot of a solution of 2.5 M NaCl or 5 mM 2,3-DPG in 50 mM bis-Tris acetate, pH 7.5, was added to the Hb sample to achieve the desired final concentration and the P_{50} was again measured at 37 °C.

Isoelectric focusing

The Hb-Resolve system from Isolabs, which employs a pH gradient, from pH 6 to 8, was used. After staining and destaining (Martin de Llano et al., 1993a, 1993b) scanning and integration of the gel bands after staining with 0.2% bromphenol blue were achieved on a Gilford model 250 spectrophotometer with a Shimadzu model CR6A integrator attached. In some studies, the separated isoelectric focusing bands were eluted from the agarose gel after cutting them separately with a razor blade and immersing them in 1 mL of CO-saturated 50 mM bis-Tris-acetate, pH 7.5. After being kept overnight on ice, the extracted Hb were recovered after centrifugation at 4,500 rpm with an Amicon Centricon in a refrigerated Sorvall RC-2B centrifuge.

Circular dichroism measurements

The CD spectra of HbA, the D99K(β) recombinant mutant, and the 2 separate bands found during isoelectric focusing were measured on an Aviv 62DS CD spectrometer equipped with a temperature controller. The instrument was calibrated with a 2-point calibration method using (+)-10-camphorsulfonic acid. Measurements were made at 20 °C and in 1-cm quartz cells on 2 different samples each of HbA, of unfractionated D99K(β) mutant Hb, and of the separate D99K(β)-1 and D99K(β)-2 forms. Wavelength scans were performed from 500 to 250 nm, digitized at 1-nm intervals, and signal-averaged with a 4-s time constant. The data were corrected for any baseline changes, smoothed, and normalized to units of molar ellipticity on a heme basis. After the analysis, the absorption spectra of the samples were recorded and their concentrations were determined by amino acid analysis after acid hydrolysis.

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